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Association of efflux pump inhibitors with antileishmanial drugs as an alternative treatment for leishmaniasis

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DISSERTAÇÃO PARA A OBTENÇÃO DO GRAU DE MESTRE EM PARASITOLOGIA MÉDICA

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Abstract

Leishmaniasis is one of the most neglected diseases in the World, according to the WHO, with new registered 300 000 cases every year. This disease affects mainly individuals from low income countries, where the access to diagnosis and treatment is very difficult. Besides this, the available chemotherapy is losing efficacy due to the emergence of resistant strains. So, there is a need for the development of new antileishmanial compounds and new strategies to refrain the impact of the disease. The proteins belonging to the ATP-binding cassette (ABC) family are transporters present in a wide variety of cells (from prokaryotes to eukaryotes) involved in the efflux of molecules. In many cases, these transporters become responsible for multidrug resistant (MDR) phenotypes and other resistance events in cells. One of the strategy to overcome this resistance is to use efflux pump inhibitors (EPI). The general goal of the present work is determine the action of efflux pumps in a context where macrophages from a cellular line (P388D1) get infected with more resistant *Leishmania* promastigotes from several species (*Leishmania infantum, Leishmania amazonensis, Leishmania shawi* and *Leishmania guyanensis*) and are exposed simultaneously to antileishmanial drugs/experimental compounds and efflux pump inhibitors. In this work, the first step was to differentiate different strains susceptible to Glucantime (GLUC), Miltefosine (MILT), ursolic acid (URS), chalcone-8 (CH8) and quercetine (QC). For the resistant strains obtained, the IC_{50} of the experimental compounds URS, CH8 and QC were then calculated. At last, in a context of macrophages infected with the different more resistant strains, it was evaluated the effect in their relative infection rate of a treatment consisting in experimental compounds combined with EPI, such as verapamil (VER), sodium orthovanadate (ORT) and Phe-Arg β-naphthylamide (PAβN). All the treatments could significantly reduce the relative infection rate of macrophages infected with the susceptible strains of *Leishmania infantum*, with the exception of the treatment with CH8 and VER in INF CH8 strain. For the several strains of *Leishmania amazonensis*, all treatments reduced the relative infection rate, with the exception for the treatments containing ORT, which seems to be harmless to this species. On the opposite side are the strains of *Leishmania shawi* and *Leishmania guyanensis,* where none of the treatments was able to reduce the relative infection rate of the macrophages. The EPI can effectively decrease the activity of efflux pumps activity and increase the efficacy

of antileishmanial drugs, and because of this, its use can be a possible alternative treatment for leishmaniasis.

Key-words: Leishmaniasis, antileishmanial compounds, resistant parasites, efflux pumps, inhibitors

Resumo

A leishmaniose é uma das doenças mais negligenciadas em todo o Mundo, de acordo com a OMS, sendo todos os anos registados 300 000 novos casos. A doença afecta, sobretudo, indivíduos de países em vias de desenvolvimento, onde o acesso ao diagnóstico e ao tratamento se torna extremamente complicado. Para além disso, a quimioterapia disponível apresenta uma eficácia progressivamente menor devido ao aparecimento de estirpes resistentes. Com tudo isto, torna-se imperativo o desenvolvimento de novos compostos antileishmania e de novas estratégias para diminuir o impacto da doença. As proteínas pertencentes à família *ATP-binding cassette* (ABC) são transportadores que se encontram presentes numa grande variedade de células (desde procariotas a eucariotas) e que são responsáveis pelo efluxo de moléculas. Muitas vezes, estes transportadores originam fenótipos resistentes, como é o caso do fenótipo *multidrug resistant* (MDR). Uma forma de ultrapassar esta resistência é recorrendo ao uso de inibidores destas bombas de efluxo. O principal objectivo deste trabalho é determinar a acção de bombas de efluxo num contexto em que macrófagos de uma linha celular (P388D1) são infectados com promastigotas mais resistentes pertences a diferentes espécies de *Leishmania* (*Leishmania infantum, Leishmania amazonensis, Leishmania shawi* e *Leishmania guyanensis*) e são expostos simultaneamente a fármacos/compostos experimentais antileishmania e inibidores das bombas de efluxo. No presente trabalho, o primeiro passo foi obter estirpes susceptíveis ao Glucantime (GLUC), à Miltefosina (MILT), ao ácido ursólico (URS), à chalcona-8 (CH8) e à quercetine (QC). Para as estirpes que se conseguiram obter, foi determinado o valor de IC_{50} para os compostos experimentais URS, CH8 e QC. Por último, num contexto em que macrófagos foram infectados com diferentes estirpes mais resistentes, foi determinado o efeito na taxa de infecção relativa de um tratamento constituído por um composto antileishmania e por um inibidor das bombas de efluxo (EPI), como o verapamil (VER), o ortovanadato de sódio (ORT) e o Phe-Arg β-naftilamida (PaβN). Todos os tratamentos foram capazes de reduzir a taxa de infecção relativa de macrófagos infectados com as estirpes susceptíveis de *Leishmania infantum*, à excepção do tratamento com CH8 e VER feito à estirpe INF CH8. Para as diferentes estirpes de *Leishmania amazonensis*, todos os tratamentos apresentaram resultados positivos, à excepção daqueles que incluíam ORT, que parece ser inofensivo para esta espécie. No extremo oposto, encontram-se as estirpes de *Leishmania shawi* e *Leishmania guyanensis*, em que nenhum dos tratamentos reduziu a taxa de infecção. Os inibidores das bombas de efluxo (EPI) conseguem reduzir a actividade das bombas de efluxo e aumentar a eficácia dos fármacos antileishmania, e, por causa disso, o seu uso pode constituir um bom tratamento alternativo para a leishmaniose.

Palavras-chave: Leishmaniose, compostos antileishmania, parasitas resistentes, bombas de efluxo, inibidores

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Abbreviation list

ABC transporters - ATP-binding cassette transporters

Cl – Cutaneous leishmaniasis

DALY - Disability-adjusted life years

DNA – Desoxyribonucleic acid

EPI – Efflux pump inhibitor

FBS – Fetal bovine serum

GUYA MILT – *Leishmania guyanensis* susceptible to miltefosine

GUYA URS – *Leishmania guyanensis* susceptible to ursolic acid

HIV - Human Immunodeficiency Virus

HOM MILT – *Leishmania amazonensis* HOM susceptible to miltefosine

HOM QC– *Leishmania amazonensis* HOM susceptible to quercetine

IC_{10} – Inhibitory concentration of 10%

IC_{50} – Half maximal inhibitory concentration

IL – Interleukin

INF CH8 – *Leishmania infantum* susceptible to chalcone-8

INF MILT – *Leishmania infantum* susceptible to miltefosine

INF QC – *Leishmania infantum* susceptible to quercetine

INF URS – *Leishmania infantum* susceptible to ursolic acid

***L.* –** *Leishmania* (subgenus)

LDA – Limit dilution assay

***Leishmania amazonensis* HOM –** *Leishmania amazonensis* isolated from a human

***Leishmania amazonensis* PH –** *Leishmania amazonensis* isolated from a phlebotomine

MCL – Mucocutaneous leishmaniasis

MDR – Multidrug resistance

MØ - Macrophage

NBD – Nucleotide binding domains

NaCl – Sodium chloride

NK – *Natural killers* cells

ORT – Sodium orthovanadate

PAβN – Phe-Arg β-naphthylamide

PH MILT – *Leishmania amazonensis* PH susceptible to miltefosine

PH QC – *Leishmania amazonensis* PH susceptible to quercetine

PH URS – *Leishmania amazonensis* PH susceptible to ursolic acid

PMN – Polimorphonuclear cells

PKDL - Post kala-azar dermal leishmaniasis

RPMI – *Roswell Park Memorial Institute culture medium*

SCHN - *Schneider's Insect Medium*

SHAW CH8 – *Leishmania shaw* susceptible to chalcone-8

SHAW MILT – *Leishmania shaw* susceptible to miltefosine

SHAW QC – *Leishmania shaw* susceptible to quercetine

SHAW URS – *Leishmania shaw* susceptible to ursolic acid

TMD – Transmembrane domains

WHO – World Health Organization

***V.* –** *Viannia* (subgenus)

v/v – volume/volume

VER - Verapamil

VL – Visceral leishmaniasis

I. Introduction

1. Epidemiology of leishmaniasis and disease burden

Leishmaniasis is one of the world's most neglected tropical diseases (WHO. 2010) and, considering both clinical manifestations of the disease, visceral and cutaneous, the reported annual cases line up to 300 000 around the world. Of these, about 58 000 cases correspond to visceral leishmaniasis and 220 000 cases to the cutaneous forms, according to data available up to 2010. However, Alvar and colleagues refer estimations that reach almost the 2 million cases every year, including 0.2 to 0.4 million of VL cases and 0.7 to 1.2 million of CL cases (Alvar et al. 2012). Considering VL, more than 90% of the reported cases occur in India, Bangladesh, South Sudan, Ethiopia and Brazil. On the other hand, CL cases are mainly distributed across Afghanistan, Iran, Pakistan, Saudi Arabia, Syria, Tunisia, Algeria, Ethiopia, Sudan, Peru, Colombia and Brazil. The concept of disability-adjusted life years (DALY) is a very useful index to assess the burden of any disease. In 2010, the estimation of DALY attributable to leishmaniasis was 3.3 million, which ranks leishmaniasis as the second most important tropical disease, only supplanted by the impact of malaria (Murray et al. 2012).

All these data concerning leishmaniasis must be faced with precaution, because it is very difficult to assess the real burden of the disease due to: (i) the focal distribution of the cases of leishmaniasis, which means that incidence is very heterogeneous within a territory, (ii) the variety of clinical manifestations, which leads to difficulties in diagnosis by medical staff and (iii) to the fact that there are different parasite species, different reservoir hosts and different vectors according to the considered region. But the main factor is the lack of available information concerning regional incidence and prevalence, DALYs, the mortality cases, as a direct result of poor organization of health services and the absence of surveillance programs (Bern et al. 2008; Singh et al. 2010). It is also well documented that conflict scenarios have an important role in the epidemiology of the disease, as it is the case of the Syrian Civil War and many other conflicts around Middle East (Jacobson. 2011). Multiple cutaneous leishmaniasis outbreaks have been happening around Syrian territory after the destruction of hospitals and other healthcare facilities (Alasaad. 2013; Alawieh et al. 2014; Hayani et al. 2015; Inci et al. 2015).

Besides that, leishmaniasis is notifiable only in 33 countries of the 98 reported as endemic, which contributes to the current state of underreporting of cases as well (WHO. 2013).

Desjeux points out some of the main factors leading to a worldwide increase of leishmaniasis incidence (Desjeux. 2001). On one hand, the expansion of deforestation and urbanization due to demographic pressure and migrations is bringing humans closer to the vectors and the reservoirs of *Leishmania* parasites. Consequently, the probability of any individual to get bitten by an infected phlebotomine will be substantially higher. One example of urbanization is the construction of dams, which always result in climate and vegetation modifications. The destruction of natural habitats has the potential to change the distribution of both sandfly (vector) and rodent (host) populations (Neouiminer. 1996). On the other hand, these processes have been occurring mainly in underdeveloped countries, which already have many problems in dealing with widespread poverty. Most of the times, poverty is associated with poor habitation, lack of sanitation and poor access to health care services. Along with that, the poor nutrition, the existence of other infectious diseases and the high cost of the available treatments for leishmaniasis also contributes to set up a scenario of increased susceptibility to leishmaniasis infection (Alvar, Yactayo, et al. 2006).

1.1 Epidemiology of visceral leishmaniasis (VL)

By the year of 2010, the World Health Organization (WHO) registered approximately 50 000 annual deaths due to the visceral form of leishmaniasis (WHO. 2010) and the main victims were children with less than 15 years old (Savoia. 2015).

Leishmania (Leishmania) donovani complex species are the responsible for this variant of the disease. *L. donovani* is associated with anthroponotic transmission of VL in the Old World, mostly rural and peridomestic foci in the Indian subcontinent, like Bihar state in India, Bangladesh, Bhutan and Nepal and foci in East Africa and Southwest Arabian Peninsula (these last in association with the zoonotic transmission of *L.(L.) infantum*). On the other hand, *L. infantum* involves peridomestic and rural foci as well, but in this case the transmission is zoonotic, occurring mainly in the Mediterranean basin, Central Asia (including Chinese regions), Saudi Arabia, Iran, Iraq and New World, more exactly in Latin America countries. It is possible to make a distinction between the cycle maintained between domestic dogs and vector and the cycle between vector and wild canines (foxes, for example). There is also evidence of

mother-to-child transmission of the parasite in humans, as some cases have already been reported (Ready. 2014).

Although the lack of consensus on the subject, South-American *L. (L.) chagasi* species are recognized to be the same as Old World *L. infantum* species. Presumably, it was the Portuguese and Spanish colonization of South America, with the subsequent migration of infected domestic dogs, that was responsible for the introduction of this species into the New World (Lukes et al. 2007).

Another important feature of this pathology is the occurrence of the post kala-azar dermal leishmaniasis (PKDL) in VL patients (caused by *L. donovani*), especially in Southeast Asia foci (Salotra and Singh 2006). Nevertheless, this region, with 100 000 VL cases estimated every year and 147 million people at risk, is achieving positive results in control and elimination of the disease: the number of cases have decreased by 59% and mortality by 89%, although new foci have been detected (WHO. 2015).

In Europe, leishmaniasis is a rare disease, with approximately seven hundred autochthonous VL cases every year, mainly in southern, western and in Balkan regions (Dujardin et al. 2008). VL is endemic in nine countries, including Portugal. According to Ready (2010), there is a risk of leishmaniasis emergence in Europe due to the introduction of exotic *Leishmania* species (through the migration of infected people from endemic areas outside Europe), the spread of *Leishmania* species to non-endemic areas where vectors of the parasite are present (mainly expansion northwards due to the movement of domestic dogs to endemic areas in a context of tourism and return to non-endemic areas) and the increase in immunosuppressed people, such as HIV infected patients or people submitted to organ transplantation. According to the World Health Organization, 70% of adult VL cases in Southern Europe occur in HIV patients (WHO. 2016b). HIV infection increases the risk of VL development, raises relapses and decreases therapeutic efficacy (Alvar et al. 2008).

The global distribution of the disease can be assessed in Fig. 1.

Figure 1. Geographic distribution of visceral leishmaniasis (or Kala-Azar) caused by *L. infantum* and *L. donovan* (red). Adapted from Chappuis et al. (2007)

1.2 Epidemiology of cutaneous leishmaniasis (CL) and mucocutaneous leishmaniasis (ML)

As it happens with VL, there are distinct vectors, reservoirs and parasite species causing cutaneous leishmaniasis (CL) according to the geographic localization. However, children with less than fifteen years old are always more susceptible to the infection, regardless the considered region (Reithinger et al. 2007).

Cl in Old World is caused by the following species: *L. (L.) major*, *L. (L.) tropica*, *L. (L.) aethiopica* and, in less extent, by *L. donovani* and *L. infantum.* CL caused by *L. major* is a rural zoonotic disease, with some wild rodents acting like a reservoir for the parasite, whereas *L. tropica* has an urban anthroponotic transmission cycle (Reithinger et al. 2007). There are registered CL cases caused by *L. infantum*, but they are inexpressive in comparison to the total amount of VL cases (Chaara et al. 2014). *L. aethiopica* has a zoonotic cycle of transmission, where hyraxes are the reservoir (Negera et al. 2008). *L. major* is present in West Africa (Senegal), Middle East and India; *L. tropica* is found in the Middle East and Maghreb and *L. aethiopica*, as its name suggests, is present in Ethiopia and, with less expression, in Kenya (Pratlong et al. 2009).

In the New World, the most relevant species causing CL are those belonging to *L. (L.) mexicana*, *L. (L.) amazonensis*, *L. (V.) braziliensis* and *L. (V.) guyanensis* complexes.

The *L. braziliensis* complex includes the homonymous species, *L. braziliensis*, and other species, such as *L. (V.) peruviana.* The cycle of transmission of these parasites is mainly zoonotic, where rural and forest environments deserve special attention. The natural reservoir hosts of *L. braziliensis* are not completely elucidated, but it is thought that small forest rodents and domestic animals (dogs, horses, donkeys) may play an important role in parasite epidemiology (Shaw. 2002; Gontijo and De Carvalho 2003). Some infected dogs have been detected, but probably they are not the main reservoir of the parasite, due to their low reservoir competence (Dantas-Torres. 2007). This species are distributed all across Central and South America.

L. guyanensis complex includes *L. guyanensis and L. shawi.* Edentate and marsupials are the natural reservoir of *L. guyanensis*, whereas monkeys and sloths maintain the cycle of transmission of *L. shawi. L. guyanensis* is mainly distributed throughout the north of the Amazon river, including some Colombia and Ecuador regions and *L. shawi* can be found south of the Amazon river (Shaw. 2002).

L. mexicana and *L. amazonensis* have some rodent species as their main hosts. *L. amazonensis* is associated with zoonotic cycles in forest environments and it is present in South America. *L. mexicana* is distributed throughout Central America and Venezuela (Shaw. 2002).

The overall geographic distribution of CL across the world can be consulted in Fig. 2.

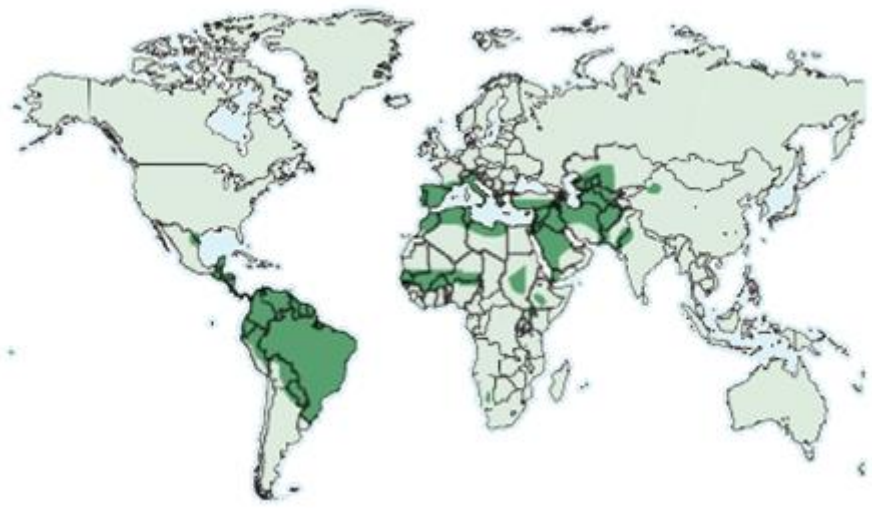

Figure 2. Endemic areas for cutaneous leishmaniasis (green). Adapted from Reithinger et al. (2007)

The main etiological agent of mucocutaneous leishmaniasis (ML) is *L. braziliensis*. However, some few cases can be caused by *L. (V.) panamensis*, especially in jungle areas or lands that were deforested. This former species has the sloth as reservoir host and occurs in Central America and Colombia (WHO. 2010)

2. Clinical presentations of leishmaniasis

2.1 Visceral leishmaniasis

Manifestations of visceral leishmaniasis (VL) or Kala-azar usually last for months or years. *Leishmania* parasites mainly infect cells that belong to the mononuclear phagocyte system. Some associated symptoms are fever, cough, diarrhea, weight loss, lymphadenopathy and, most important, a progressive hepatosplenomegaly and bone marrow suppression. Development of these symptoms are followed by pancytopenia and immune-suppression and, ultimately, death overcome in two years after infection if no treatment is administered (Kevric et al. 2015)

2.2 Cutaneous leishmaniasis

Usually, the incubation period of cutaneous leishmaniasis can take days to months. The clinical manifestations always begin with a small papule that can ulcerate in cases of infection by *L. major* or New World cutaneous species, or, alternatively, can evolve to nodules or go through a process of hyperkeratosis (dry lesions) (Bailey and Lockwood. 2007). Nodular lesions usually appear in infections by *L. aethiopica* and by the species of the *L. donovani* complex, whereas hyperkeratotic lesions occur in the context of *L. tropica* infection. These lesions imply pain, pruritus and, in some cases, secondary bacterial infections. Another variant of acute CL includes local dissemination of parasites or antigens to the surrounding regions of the original lesion, in particular through the lymphatic vessels.

The disease acquires a wider disseminated character when ten or more lesions occur in two or more nonadjacent areas of the body (Bailey and Lockwood. 2007). This type of cases is caused by New World species and is very rare.

Diffuse CL consists in the development of nonulcerating lesions followed by dissemination to the face and exterior surfaces of the limbs and the eventuall destruction

of deeper tissues. This clinical form of the disease it is associated with *L. amazonensis* and *L. aethiopica* infections (Herwaldt, 1999).

2.3 Mucocutaneous leishmaniasis

MCL is initially characterized by the development of local CL lesions and the appearance of parasites metastasis. In the following stage, the parasites disseminate through hematogenous or lymphatic spread and invade the mucocutaneous tissue, such as the nose, the mouth and the noropharyngeal mucosa. This form of the disease can last months to several years. The chronic symptoms consist in the progressive destruction of the noropharyngeal mucosa, which leads to the disfiguration of the affected individual (Mcgwire and Satoskar. 2014). Along with this, the respiratory function and the nutrition are hampered.

3. The phlebotomine vector

Phlebotomines are insects included in Order *Diptera*, family *Psychodidae* and subfamily *Phlebotominae*. Until now, the blood-feeding phlebotomine females are the only proven natural vectors of *Leishmania* parasites (Ready. 2013). There is not a consensus about the exact number of sandfly species that exist in the world. For example, Ready cites approximately nine hundred different species, seventy of which implicated in leishmaniasis transmission (Ready. 2013). Marolli and collaborators refer eight hundred species and ninety eight species involved (or suspected to be involved) in leishmaniasis transmission (Maroli et al. 2013). Of these, there are forty-two Old-World phlebotomine species, more specifically those that belong to *Phlebotumus* genus, and there are fifty-six New-World species, belonging to *Lutzomyia* genus. However, looking to older articles, the numbers that can be found may be very different from these one: Killick-Kendrick points out eighty-one species of sandflies and nineteen species proven vectors of *Leishmania* parasites (Killick-Kendrick. 1990). This lead to the conclusion that information can vary according to the taxonomic classification that is used.

In Portugal, five different phlebotomine species are present: *Phlebotomus (Larrousius) perniciosus* Newstead, 1911, *P. (L.) ariasi* Tonoir, 1921, *P. (Paraphlebotumus) sergenti* Parrot, 1917, *P. papatasi* Scopoli, 1786 and *Sergentomyia (Sergentomyia) minuta* Rondani, 1843 (Branco et al. 2013). However, only two species, *P. perniciosus* and *P. ariasi*, have been implicated in leishmaniasis transmission, more

precisely in the transmission of *L. infantum* (Pires. 1984 cited by Campino and Maia. 2010). *P. sergenti* and *P. papatasi*, vectors of *L. tropica* and *L. major*, respectively, have been detected in Portuguese territory, in the southern region of Algarve (Maia et al. 2009), but there are not reported autochthonous cases of leishmaniasis caused by the referred parasites. Several studies have been demonstrating the presence of sand fly species all across the country, where some foci, due to their association with canine and human leishmaniasis, deserve special attention: Alto-Douro (Afonso et al. 2007), Lisbon Metropolitan Region (Afonso et al. 2005), Évora region (Afonso and Semião-Santos 2004) and Algarve region (Maia et al. 2009).

4. Parasite life cycle

The life cycle of parasites belonging to *Leishmania* genus includes two different developmental stages (dimorphic life cycle): one that occurs inside a phlebotomine vector; another that happens inside a vertebrate host, which can be a human, a dog, a rodent, or another species. The complete schematic of the life cycle can be assessed in Fig. 3.

The parasite itself can assume two distinct forms: a promastigote stage, a motile, elongated form with 5 μm in diameter; an amastigote stage, a non-flagellated, spherical form with 2.5 to 5 μm in diameter.

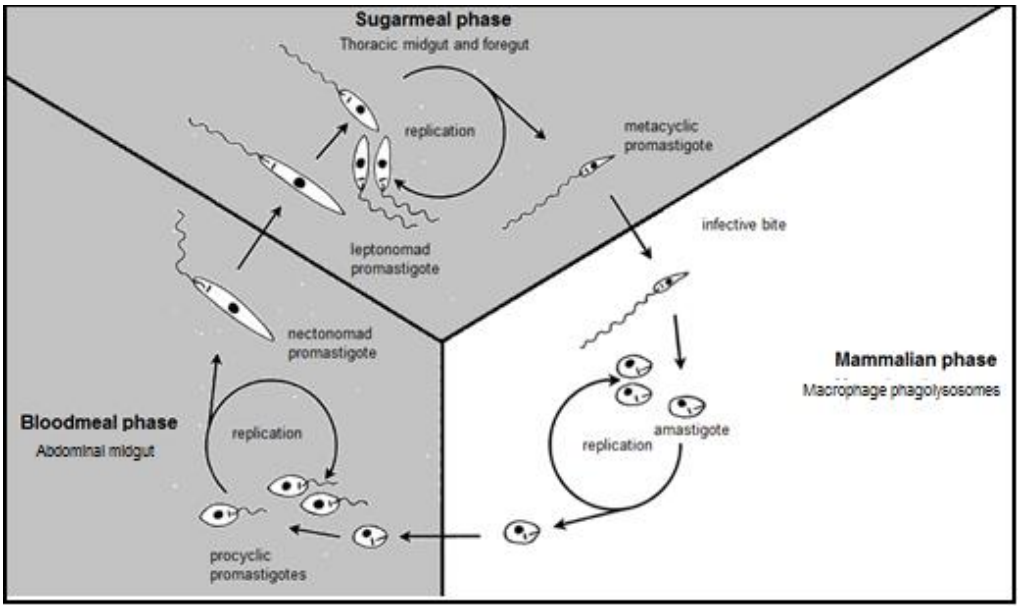

Figure 3. Schematic life cycle of the parasites belonging to *Leishmania* genus. Grey area: development in sand fly vector. White area: development in vertebrate mammalian host. Adapted from Gossage et al. (2003).

4.1 Vector stage

During its life cycle, the parasite thrives in diverse environments with different conditions of temperature, pH and others. An essential feature of this cycle happens when a female phlebotomine takes a blood meal in the infected vertebrate host through pool feeding. This process involves the cutting of the host skin with the mouthparts of phlebotomine, followed by feeding from the resulting blood pool. Together with the blood meal, aflagellated amastigotes are also ingested; these forms will divide in the midgut of the vector, experiencing a colder and more alkaline environment than the environment provided by the vertebrate host. *Leishmania* parasites increase the expression of surface molecules, such as the glycoconjugates lipophosphoglycan (LPG) and metalloprotease gp63, to enable the survival in the hydrolytic environment of the gut (Cunningham. 2002). Simultaneously with division, amastigotes are converted in procyclic promastigotes, little motile parasites. This blood meal phase occur within the peritrophic membrane, a chitinous matrix secreted by the epithelial cells of the gut (Bates. 2007). One of the criteria to differentiate the subgenus *Leishmania* and the subgenus *Viannia* is to consider the specific site that parasite occupy in the sandfly gut: parasites of subgenus *Leishmania* are present in the midgut and foregut of the vector, whereas parasites of subgenus *Viannia* in the midgut, the foregut and also the hindgut (Gossage et al. 2003). After some days post-infection and before the complete digestion of the blood meal, the parasites convert into nectonomads, a migratory form, and accumulate in the anterior abdominal midgut, while producing and secreting promastigote-secretory gel (PSG) (Bates. 2007). This accumulation ultimately leads to the destruction of the peritrophic matrix and to the following release of blood medium. Promastigotes then migrate to thoracic midgut and to stomodeal valve, where they originate leptonomads, shorter forms that replicate and later convert into metacyclic promastigotes (high motile forms), the unique infective form to the vertebrate host; some of the migratory nectonomads also convert into a shorter and circular form called haptomonads (Schlein. 1993).

There are two alternate views on the transmission of metacyclic promastigotes to the vertebrate host: inoculation versus regurgitation. Inoculation theory says that only the metacyclic promastigotes present in sandfly proboscis are transmitted to the vertebrate host during the bite. On the other hand, the “blocked fly hypothesis” claim that the obstruction and damage of stomodeal valve lead to the reflux of parasites during the bloodmeal and consequent infection of the host (Bates. 2007). There is no consensus

on the subject, but it is thought that the two processes may occur simultaneously or independently according to the *Leishmania* species or the sandfly species considered.

4.2 Vertebrate stage

As a female phlebotomine takes a blood meal in the vertebrate host, simultaneously inoculates metacyclic promastigotes into the skin, which promptly elicit the immune response of the victims. Polimorphonuclear cells (PMN) and mononuclear phagocytic cells are the first to act in the region of the bite, which favors the progression of infection. Neutrophils, macrophages and dendritic cells are phagocytic cells able to internalize the metacyclic promastigotes.

As Laskay and collaborators refer (Laskay et al. 2003), neutrophils can be considered «Trojan horses», because they indirectly allow the entrance of microorganisms into the macrophages. For example, it has been proved that the species *L. major, L. aethiopica* and *L. donovani* have a marked chemotactic effect on human PMN, but not in macrophages and *Natural killer* (NK) cells (Van Zandbergen et al. 2002). This effect is achieved through the production of a chemotactic factor by the parasites and, simultaneously, through the induction of interleukin (IL)-8 (a chemokine) production by PMN. Neutrophil apoptosis and subsequent ingestion by macrophages are natural mechanisms of the inflammatory response (Witko-Sarsat et al. 2000). It follows that *Leishmania* infected neutrophils are ingested by macrophages, thus favoring parasite survival. First, because the macrophage receptors are not involved in this process, the pathways that depend on these interactions leading to pathogen elimination are not activated. On the other hand, the phagocytosis of apoptotic neutrophil will have an immunosuppressive effect in the macrophage (Sun and Shi. 2001), contributing to proliferation of the parasite within this cell. Besides this, *Leishmania* parasites have the capacity to interfere with receptor responsiveness in macrophages, such as the Toll-like receptor and CD40 (Bhardwaj et al. 2010).

In the intracellular environment of macrophages, the parasitic form (whether it is a metacyclic promastigote or an amastigote) will initially thrive in the parasitophorous vacuole. This compartment later fuses with early and late endosomes and lysosomes, a process that originates a phagolysosome with very acidic conditions and high temperature (Liévin-Le Moal and Loiseau. 2016). Then, the metacyclic promastigotes convert into non-motile amastigotes. During the intracellular stage, the parasite

modulates the host cell pathways and subvert its defense mechanisms against pathogens, such is the case of oxidative damage, to ensure its survival and replication (Moradin and Descoteaux. 2012). This stage ends with the release of the amastigotes to the extracellular space, either by cell lysis due to excessive parasite replication or to the active manipulation of the exocytic pathways of the host cell by the parasite (Rittig and Bogdan. 2000). From here, amastigotes can infect other cells in different organs (skin or deeper tissues, such as the liver and the spleen), depending on parasite species and host susceptibility. Amastigotes are then available in the bloodstream and, if a phlebotomine takes a bloodmeal in the infected individual, the parasite will be transmitted to the vector and therefore the cycle will continue.

5. Treatment of leishmaniasis

In general, the main problems arising from the chemotherapy application in clinical cases are related with high toxicity and subsequent adverse reactions, long duration of treatment leading to a decrease of compliance and to the fact that most drugs do not eliminate completely the parasite from the organism (Menezes et al. 2015).

5.1 Treatment of visceral leishmaniasis

Pentavalent antimonials have been the standard first-line medicines for the treatment of visceral leishmaniasis for decades. Sodium stibogluconate (Pentostam®) and meglumine antimoniate (Glucantime®) are the two chemically similar forms used in clinical field against a variety of *Leishmania* species (Piscopo and Mallia Azzopardi. 2007).

Thiol redox homeostasis is absolutely vital to the survival of parasite, because it has an impact on parasite response against chemical and oxidative stress (Baiocco et al. 2009). Trypanothione ($T(SH)_2$), an enzyme only present in trypanosomatids, is the mainstay of this system. Its production requires the synthesis of glutathione (GSH) and spermidine (Spd) and the further conjugation of these metabolites catalysed by trypanothione synthetase (TryS), as it is visible in Fig. 4 (Leroux and Krauth-Siegel. 2015).

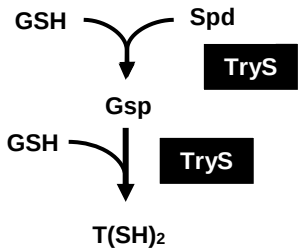

Figure 4. – Simplified overview of trypanothione synthesis. Abbreviations: GSH, glutathione; Spd, spermidine; Gsp, glutathionylspermidine; TryS, trypanothione synthetase; $T(SH)_2$, trypanothione (reduced form).

Trypanothione reductase (TR) is responsible for linking the NADPH based metabolism to thiol based metabolism, as it catalyses the reduction of trypanothione using a NADPH molecule (Fig.5).

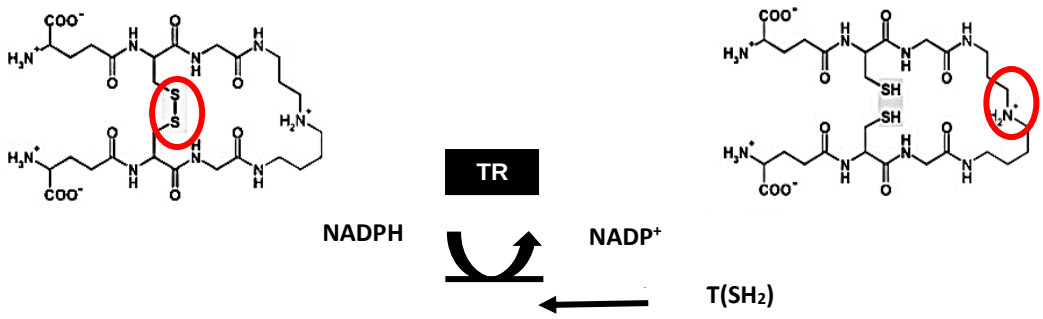

Figure 5. – Activity of trypanothione reductase. In the presence of NADPH, trypanothione reductase reduced the dissuphide bound of trypanothione originiating of the reduce form of trypanothione. Abbreviations: TS_2, trypanothione disulphide; TR, trypanothione reductase; $T(SH)_2$, trypanothione (reduced form); $NADP^+$, nicotinamide adenine dinucleotide phosphate; NADPH, reduced form of $NADP^+$.

Although their profuse application, the molecular and cellular mechanisms of action of pentavalent antimonials are not completely unveiled and, consequently there are different theories trying to explain the leishmanicidal action of this class of compounds. Pentavalent antimonials are pro-drugs, which means they need to be reduced to trivalent form to be activated. Thiol-dependant reductase (TDR1) is an enzyme present in higher concentrations in the amastigote stage of *Leishmania* and have

the capacity to convert Sb(V) in its reduced form, Sb(III) (Denton et al. 2004). This is probably the reason why amastigotes are more susceptible to antimonials action than promastigotes.

It is thought that the reduced form of pentavalent antimonials, or Sb (III), can interfere with host immune activation, inducing oxidative and nitrosative stress in macrophages. On the other hand, these drugs can affect trypanothione metabolism of the parasite itself through many ways. First, they can stimulate the rapid efflux of intracellular trypanothione and glutathione; second, they inhibit trypanothione reductase action, which causes the accumulation of disulfide forms of both trypanothione and glutathione. This ultimately compromises the thiol redox potential of parasite, leading to its death (Leroux and Krauth-Siegel. 2015; Wyllie et al. 2004). Since molecules like trypanothione reductase and trypanothione are specific for trypanosomatids, they can be good targets to the development of new chemotherapeutic agents.

These compounds are administered through intramuscular or intravenousl injections and have been associated with severe adverse reactions like anorexia, vomiting, nausea, abdominal pain, malaise, myalgia, headache and lethargy (WHO, 2010). Although the easy availability and low cost of these drugs, the length of treatment is often a problem. Jointly with the generally decrease of efficacy, mainly due to emergence of parasitic resistant-strains, these factors restrain its use in the clinical field. Resistance to treatment has been documented all around the world, particularly in Indian subcontinent: in hyper endemic northern region of Bihar it was registered a pentavalent antimony resistance of 65% in a group of treated patients (Sundar et al. 2000).

Amphotericin B (deoxycholate) is a polyene antibiotic that originally was exclusively used in the treatment of fungal infections (Hamill, 2013). However, this drug also have efficacy against a variety of other micro-organisms, like *Leishmania* and *Trypanosoma cruzi* (Croft et al. 2006). It has been a second-line drug for the treatment of visceral leishmaniasis, especially in antimony-resistant cases. However, in India, it is included in first-line drugs, due to widespread resistance to pentavalent antimonials (Croft and Olliaro, 2011). The compound binds ergosterol present in cell membranes, which consequently leads to the formation of membrane channels and ultimately cell disruption (Croft et al. 2006). The high toxicity and adverse reactions associated with the application of this drug in clinical cases, like fever, rigor, chills, thrombophlebitis of the injected vein and nephrotoxicity (WHO, 2010) has been limiting its use. To

overcome these obstacles, new formulations of the drug have been developed. Liposomal amphotericin B seems to be a viable alternative because it can decrease the adverse effects associated with drug administration and, simultaneously, improve drug pharmakinetics and bioavailability (Hamill, 2013), although it has an high cost. Pentamidine (1,5-bis(4-amidinophenoxy)pentane) is an antimicrobial drug that is included in second-line treatment of visceral leishmaniasis. Pentamidine and its analogues have been used for nearly sixty decades in the treatment of various diseases besides leishmaniasis, like malaria, human african trypanosomiasis and *Pneumocystis carinii* pneumonia (Porcheddu et al. 2012). Its mechanism of action is not well understood, but, as trypanosomes have the capacity of actively internalize pentamidine, it is thought that the drug could affect DNA biosynthesis of parasite (Sands et al. 1985). Intramuscularly injection, secondary effects like diabetes mellitus, hypoglucaemia, shock, miocardis nephrotoxicity and low efficacy do not encourage a more intensive use of the drug (WHO 2010).

Miltefosine was registered for commercial use in 2002 and at the moment is the only oral drug available for the treatment of both visceral and cutaneous leishmaniasis. Miltefosine (or hexadecylphosphocholine) is a compound belonging to alkylphospholipids family that was initially used in the treatment of tumours. Its mechanism of action consists in the induction of apoptosis-like cell death and dysregulation of lipid metabolism (Dorlo et al. 2012a). Rakotomonga et colleagues achieved evidence of alterations in phospholipidic membrane of the *L. donovani* parasites after miltefosine exposure, specifically “intromission” of molecules of hexadecylphosphocholine in phospholipid monolayer and decrease in phospatidylcholine content simultaneously with increase of phosphatidylethanolamine content (Rakotomanga et al. 2007).

5.2 Treatment of cutaneous leishmaniasis

Many of the chemotherapeutic agents available in the treatment of visceral leishmaniasis have application in the treatment of the cutaneous form of leishmaniasis. The disease is usually self-limiting, not fatal and therefore the application of therapy is mainly local.

Pentavalent antimonials are used in CL treatment through intralesionally administration. Their efficacy in not completely proven yet, as there is a significant

variation in clinical response between the different *Leishmania* species. Amphotericin B deoxycholate is a second-line drug in CL treatment, but the cost, the need for parental administration and the toxicity associated hampers its use (Alvar and Croft, et al. 2006). Miltefosine is also used in Cl treatment, however its use is not effective in all relevant *Leishmania* species. For example, Soto and colleagues showed that this drug is only active in the treatment of CL caused by *L. panamensis* and not CL caused by *L. braziliensis* (Soto et al. 2004). Paromomycin is present in various topical formulations, being a useful drug against both old and new cutaneous leishmaniasis. WHO recommends a topic containing 15% paromomycin/12% methylbenzethonium chloride for the treatment of Cl caused by *L. major, L. tropica, L. aethiopica, L. infantum* and all forms of New World CL (WHO, 2010).

5.3 Development of new potential chemotherapeutic agents

Flavonoids (included in the group of polyphenols) are a class of secondary metabolites mainly extracted from plants, like fruits, vegetables, nuts, stems, flowers and also wine and tea, being part of the daily diet of the human being (Scalbert and Williamson 2000).

Chalcones are members of this group and their relatively simple structure and preparation allows the synthesis of derivatives with different biological functions (Passalacqua et al. 2015). Some of these derivatives may present relevant leishmanicidal activity, which justifies their study and development.

The standard structure of chalcones comprises an open chain with two aryl rings (an aryl ring is a substituent group made of an aromatic ring or one of its derivatives) connected by an α,β – unsaturated carbonyl structure, as it is visible in Fig.6 (Roussaki et al. 2013).

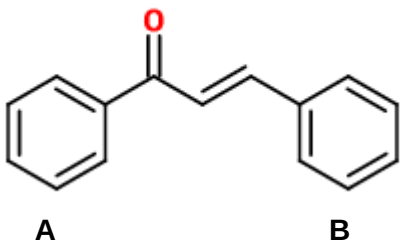

Figure 6. General structure of Chalcones, with two aryl rings: A and B. From https://www.emolecules.com/

There is some evidence that chalcones and their derivatives can have an important role in the fighting against *Leishmania* species. Litochalcone, for example, inhibit in vitro growth of *L. major* and *L. donovani* promastigotes and kills *L. major* amastigotes (Zhai et al. 1995). This happens because litochalcone destroy the parasitic mithocondria, more specifically, the respiratory chain of the parasite, thus impairing its respiratory activity and its survival. Another derivative of chalcone, 2',6'-DIhydroxy-4'-methoxychalcone (DMC) have shown selective *in vitro* activity against promastigote and amastigote forms of *L. amazonensis* and no activity against macrophages (Torres-Santos et al. 1999). Chalcones (1-4), derivatives of the general structure of chalcones, reduce significantly the parasite burden of *L. braziliensis* in macrophages, without having a cytotoxic effect on these cells (de Mello et al. 2014).

Quercetin, a plant-dietary flavonoid, is another compound that has shown interesting anti-leihsmanial activity. This compound is a flavonol, which is a class of compounds that share a 3-hydroxyflavone backbone (Fig.7).

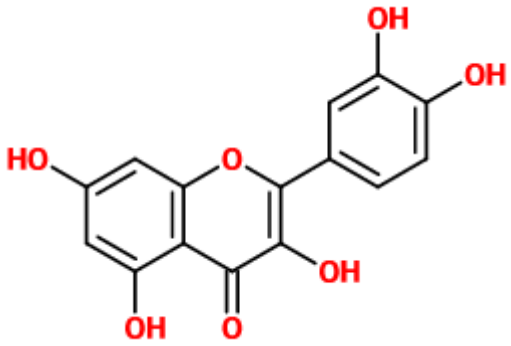

Figure 7. Structure of quercetin. From https://www.emolecules.com/

Quercetin can impair *in vitro* growth of both *L. donovani* promastigotes and amastigotes, according to Mittra and colaborators (Mittra et al. 2000). They have demonstrated that quercetin and luteolin (another flavonoid) interphere with *Leishmania* topoisomerases activity, which led to significant promastigote apoptosis and reduction of parasite burden in the spleen of hamsters. Besides this, quercetin is also implied in the inhibition of *L. amazonensis* arginase (Da Silva et al. 2012). Arginase is an enzyme involved in the catalisation of the final step of urea cycle and quercetin can compete with L-arginine and Mn^{2+}, substrate and cofactor, to the binding site of this enzyme.

Iron is essential for *Leishmania* survival inside macrophage phagolysosomes, because the parasite can't synthetize the heme group, so it needs to use the iron of the

host. Quercitin is a lipophilic metal chelator, which means that it binds to metal ions through hydrogen binding and can cross the cellular plasma membrane. Sen and collaborators provided a combined treatment of quercetin and serum albumin to hamsters infected with *L. donovani*, which led to significant reduction of splenic parasite burden (Sen et al. 2008). This happens due to interpherence in parasite iron metabolism, more specifically the reduction of ribonucleotide reductase activity in phagolysosomes, which is an iron-dependent enzyme involved in DNA synthesis.

Ursolic acid is a triterpene that is present in food and in many natural plants. This pentacyclic triterpenoid, which structure is represented in Fig.8, has some therapeutical application, for example, in the apoptosis of tumor cells (Wang et al. 2011).

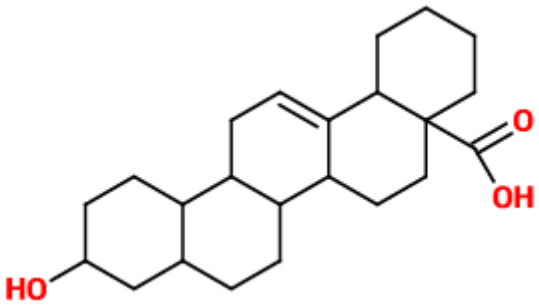

Figure 8. Structure of ursolic acid. From https://www.emolecules.com/

In addition, other effects of this compound have been documented, with particular attention to the antiprotozoal ones. Ursolic acid extracted from *Baccharis dracunculifolia*, a plant belonging to *Asteraceae* family, has shown interesting leishmanicidal activity (IC_{50} = 3.7 $\mu g.ml^{-1}$) against the promastigote forms of *L. donovani* (Filho et al. 2009). Passero and coworkers have demonstrated that a fraction containing oleanolic and ursolic acid significantly decreases the growth rate of *L. amazonensis* and *L. braziliensis* amastigotes and decreases the infection rate of J774 macrophages, due to the increase in nitric oxide production (Passero et al. 2011). These antileishmanial effects were accompanied by a lack of citotoxicity in macrophage cells. Other *in vivo* studies corroborate these *in vitro* findings, as it is the case of Yamamoto et al. (2014). In this work, BALB/c mice infected with *L. amazonensis* were treated with a triterpenic fraction composed by oleanolic and ursolic acid and the results were comparable to those obtained when the mice were treated with amphotecin B. The same

group have proved that ursolic acid alone is capable of destroy *L. amazonensis* promastigotes with a dose comparable to miltefosine, while lacking toxicity towards peritoneal macrophages of BALB /c mice (Yamamoto et al. 2015). According to the authors, the promastigotes were killed by programmed cell death related with mitochondrial activity and the amastigotes due to the increase in nitric oxide production by macrophages.

6. The role of ABC transporters in drug resistance

At cellular level, any molecule, ion, drug or virus needs to cross biological membranes, with the ultimate goal of ensuring the survival of any cell or, if it's the case, the survival of some pathogenic micro-organism. One of the main class of proteins involved in the translocation of particles across membranes is the ATP-binding cassette transporters (ABC transporters) superfamily, which is widely represented in both prokaryote and eukaryote domains. In prokaryotes, these transporters are responsible for the import and extrusion of substrates, while in eukaryotes cells the ABC transporters only participate in extrusion (Sauvage et al. 2009). For example, in human cells, these proteins are involved in transportation of endogenous substrates, as inorganic anions, metal ions, peptides, amino acids, sugars and a large number of hydrophobic or cytotoxic compounds and metabolites across the plasma membrane (Vasiliou et al. 2009).

Most of eukaryote ABC proteins are a polypeptidic chain composed of four domains: two hydrophobic transmembrane domains (TMD), responsible for substrate translocation, and two nucleotide-binding domains (NBD), involved in ATP binding and hydrolysis (usually represented by TMD_2-NBD_2) (Fig.9). The nucleotide-binding domains are more conserved throughout the species, due to the presence of three consensus regions: Walker motifs A and B (involved in magnesium-ATP binding) and an ABC transporter signature, the "C motif", that is located between the two Walker motifs and has unknown function (Pérez-Victoria et al. 2001)

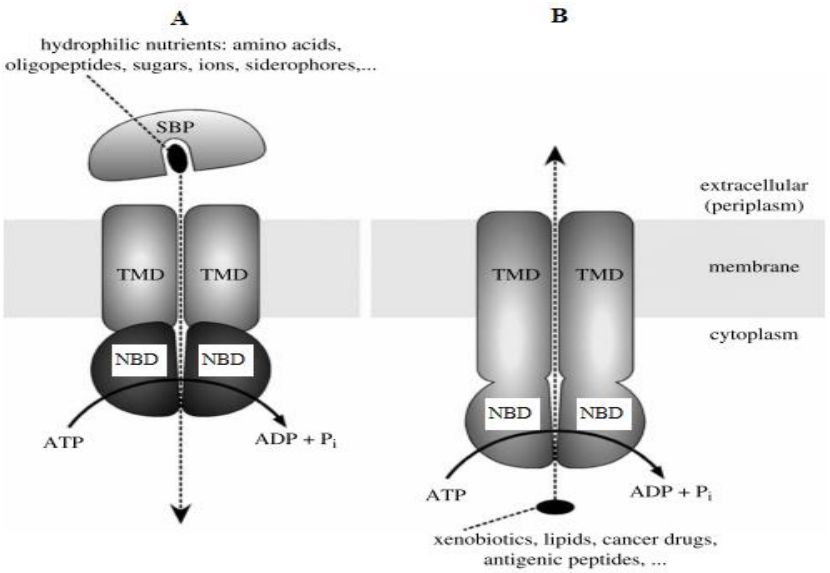

Figure 9. ABC transporters involved in import (A) and export (B) of substrates. TMD represents the transmembrane domains; NBD, nucleotide binding domains, bind the adenosine-triphosphate (ATP) molecules. The resulting hydrolysis of ATP provides the necessary energy for the translocation of substrates across the plasma membrane, with the subsequent production of adenosine diphosphate (ADP) and inorganic phosphate (P_i). Adapted from Locher (2009)

The genome of *Leishmania* spp contains 42 genes belonging to ABC genes family, but only 32 encode ABC transporters proteins. It is the largest ABC data set among protozoans (Sauvage et al. 2009). The ABC transporters superfamily are divided into 8 subfamilies, according to gene structure similarity and NBD homology. Every single one of the 8 major subfamilies that can be encountered in eukaryotic cells are present in *Leishmania* genome (from ABCA to ABCG subfamily) (Leprohon et al. 2006).

The first subfamily is ABCA, which comprises ten genes of the *Leishmania* genome. This set of genes encodes ABC functional transporters with TMD_2-NBD_2 topology. The first member of this subfamily to be discovered was LtrABC1.1 and it is involved in lipid movements across the plasma membrane and in vesicle trafficking; transfected *L. tropica* promastigotes overexpressing this transporter showed a decrease in infectivity of macrophages (Parodi-Talice et al. 2003). The other ABCA transporter characterised, LtrABCA2, seems to have the same functions of LtrABC1.1 (Araújo-Santos et al. 2005), which means that none of these proteins is related to drug resistance in *Leishmania* spp.

The second subfamily is ABCB and the *Leishmania* genome contains four genes

belonging to this group. The expression of the P-glycoprotein transporters in mammalian cells confers a multi-drug resistance (MDR) phenotype, specifically in cancer cells. In *Leishmania*, some P-glycoprotein-like transporters are expressed. Two of these genes encode full-transporters with TMD_2-NBD_2 topology that are present in subcellular locations: ABCB4 (mdr1 homologous) and ABCB2 (mdr2 homologous). Henderson and coworkers first demonstrated that amplification of *ldmdr1* gene in *L. donovani* was responsible for a drug-resistant phenotype (Henderson et al. 1992a). Later, it was found out that the homologous of this gene in *Leishmania tropica* was located in extrachromosomal circular DNA: expression of this P-glycoprotein was increased in parasites resistant to daunomycin (Chiquero et al. 1998). Since then, many works have been clarifying the association between these Pgp-homologues and resistance to various drugs in some *Leishmania* species, such as vinblastine (Dodge et al. 2004), doxorubicin and actinomycin D (Katakura et al. 1999) and, more important due to their application in the clinical field, miltefosine (Pérez-Victoria et al. 2001). On the other hand, the ABCB2 (or MDR2) transporter is associated with resistance to 5-fluoroacil (a drug used in cancer chemotherapy) in wild-type promastigotes belonging to *L. amazonensis* species (Katakura et al. 2004a).

The third subfamily, ABCC, is also represented in *Leishmania* genome with eight members. Six proteins encoded by these genes are homologous to the members of multidrug resistance-associated proteins (MRP) found in mammalian cells. The gene *LtPGPA* (or *ABCC3*) that encodes P-glycoprotein A (a MRPA homologous), or simply PGPA, was first discovered in H-circles (extrachromosomal DNA) of methotrexate-resistant parasites belonging to *L. tarentolae* species (Ouellette et al. 1990). In fact, this transporter has an important role in parasite resistance to compounds containing heavy metals, such as arsenite and antimony (Callahan and Beverley. 1991; Singh et al. 2014).This protein is positioned in membranes next to the flagellar pocket of the parasite and acts through the sequestration of metal-thiol conjugates (containing glutathione or trypanothione) into a intracellular vesicle, thereby avoiding toxic effects (Légaré et al. 2001). Another transporter belonging to this subfamily is pentamidine resistance protein 1 (PRP1) and, as the name reveals, has been associated with resistance to pentamidine and trivalent antimonials in both *L. major* promastigote and amastigote forms (Coelho et al. 2003, 2007). This transporter is only present in *Leishmania* genus and not in other trypanosomatids, such as those included in *Trypanosoma* genus (Leprohon et al. 2006). However, the role of this protein in

resistance seems to be restricted to some *Leishmania* species because it has been proved that laboratory-induced resistance does not increase PRP1 expression in *Leishmania amazonensis* promastigotes (Coelho et al. 2008).

The ABCG subfamily has six genes in *Leishmania* genome, all encoding half-full transporters, i.e., proteins with NDB-TMD topology. These proteins require dimerization to assemble a functional protein that can be a homo- or a heterodimer. The main proteins of this subfamily are ABCG6 homologues, like LiABCG6 and LdABCG6, and ABCG4 (LiABCG4). Their function is related with lipid transport across membranes, more specifically short-chain phospholipids analogues, and with extrusion of multiple compounds, such as alkyl-phospholipids (miltefosine, perifosine, edelfosine), sitamaquine, camptothecin. (Castanys-Muñoz et al. 2007; BoseDasgupta et al. 2008; Castanys-Muñoz et al. 2008). To accomplish this, the position of these proteins in plasma membrane and flagellar pocket region are essential factors.

Until this moment, proteins encoded by genes of ABCD, ABCE, ABCF e ABCH subfamilies are not characterised in *Leishmania* spp, although there are ten genes included in these groups. Another four ABC genes not included in any subfamily are present in the parasite genome (Sauvage et al. 2009).

7. Modulation of ABC transporters in *Leishmania* spp

The failure of traditional chemotherapeutic agents against the clinical forms of leishmaniasis urges the need of alternative strategies in the treatment of the disease. The use of drugs or new leishmanicidal compounds associated with ABC transporter modulators, mainly inhibitors, is a promising way to accomplish that objective. The main modulators are divided in various groups: calcium channel blockers, flavonoids, sesquiterpenes and pyridine analogues (Pradines et al. 2005).

The calcium channel blockers, as the name implies, act through the impairment of the activity of efflux pumps relying on calcium pathways. For example, verapamil (VER), widely used in the treatment of heart diseases, is a well-known inhibitor of the P-glycoprotein activity in cells with MDR phenotype (Wu et al. 2014), specifically in tumour cells (Tsuruo et al. 1981; Rogan et al. 1984). Neal and co-workers were the first to explore the use of verapamil in the reversal of drug-resistance phenotypes in trypanosomatids, more specifically in nifurtimox-resistant *T. cruzi* and antimony-resistant *L. donovani* (Neal et al. 1989). The energy-dependent efflux of pirarubicin, a drug that inhibits DNA replication, is inhibited by verapamil in *L. mexicana*, *L.*

guyanensis and *L. braziliensis* promastigotes (Essodaïgui et al. 1999). Besides this, many phenothiazine derivatives are included in calcium channel blockers. Phenothiazine derivatives inhibits the binding of calcium to calmodulin (transport protein) and thus affect the efflux pump activity (Grácio et al. 2003). These compounds have antimicrobial activity and successfully revert both multi-drug resistant *Mycobacterium tuberculosis* (MDRTB) and methicillin-resistant *Staphylococcus aureus* (MRSA) phenotypes (Amaral et al. 2004) and have antimalarial activity and can revert drug-resistance phenotype in chloroquine resistant *Plasmodium falciparum* (Guan et al. 2002). In *Leishmania spp,* it was previously demonstrated that thioridazine, prochlorperazine, trifluoperazine, chlorpromazine and trifluoropromazine inhibit energy-dependant efflux of pirarubicin in resistant parasites from *L. braziliensis*, *L. mexicana* and *L. guyanensis* species (Essodaïgui et al. 1999).

II. Aims

The general aim of the present study is to evaluate the action of efflux pumps in a context of P388D1 macrophages infected with *Leishmania* spp parasites previously exposed to conventional antileishmanial drugs, antileishmanial experimental compounds and efflux pump inhibitors.

1. Specific aims

2. To differentiate promastigotes of several different strains of *Leishmania* spp more resistant to commercial antileishmanial drugs and experimental antileishmanial compounds.
3. To determine the antileishmanial effect of experimental compounds in several resistant *Leishmania* spp strains.
4. To characterize the effect of the association of antileishmanial compounds with efflux pump inhibitors (EPI) in a context of macrophage (P388D1) infection by more resistant *Leishmania* spp promastigotes.

III. Materials and methods

1. Parasites

1.1 - Culture of Leishmania *promastigotes*

Promastigotes were cultivated in *Schneider's Insect Medium* (SCHN, Sigma Aldrich) with L-glutamine, supplemented with 10% (v/v) Fetal Bovine Serum (FBS, Biowest), 100 $\mu g.mL^{-1}$ of streptomycin (Sigma-Aldrich) and 100 $U.mL^{-1}$ of penicillin (Sigma-Aldrich) and pH 7.2. The cultures were maintained at 24 ºC. The strains referred below were used in this work.

1.2 - Leishmania (L.) infantum

L. infantum (MCAN/PT/2012/IMT0005SG) was obtained from a canine leishmaniasis case in the municipality of Seixal, Setúbal, Portugal and maintained in BALB/c mice. Virulent promastigotes were isolated from the spleen of the infected mice and inoculated into the culture medium. These promastigotes were maintained in culture until four passages in order to retain their virulence.

1.3 - Leishmania (L.) amazonensis

In this work were used two different strains of *L. a*mazonensis: HOM/BR/1973/M2269 e IFLA/BR/67/PH8.

The strain HOM (MHOM/BR/1973/M2269) was isolated from a patient with American tegumentary leishmaniasis in the state of Pará, Brazil. It was classified by monoclonal antibodies and isoenzymes in Evandro Chagas Institute, Belém, State of Pará, Brazil. This strain was provided by Luiz Felipe Passero, from *Laboratório de Patologia de Moléstias Infeciosas, Faculdade de Medicina da Universidade de São Paulo,* Brazil (LPMI-FMUSP).

The strain PH (IFLA/BR/67/PH8) was isolated in a phlebotomine collected in the state of Pará, Brazil. It was classified by isoenzymes in Evandro Chagas Institute, Belém, State of Pará, Brazil. This strain was provided by Liliane Rocha, from *the Laboratório de Leishmaniose e Doença de Chagas, Instituto Nacional de Pesquisas da Amazónia,* Manaus, Brazil (LLDC-INPA).

1.4 - Leishmania (V.) shawi

This strain (MHOM/BR/96/M15789) was isolated from an individual with cutaneous leishmaniasis in Buriticupu, State of Maranhão, Brazil. These parasites were maintained in BALB/c mice footpad and then isolated. This strain was classified monoclonal antibodies and isozenzymes in Evandro Chagas Institute, Belém, State of Pará, Brazil. This strain was provided by Luiz Felipe Passero, from *Laboratório de Patologia de Moléstias Infeciosas, Faculdade de Medicina da Universidade de São Paulo*, Brazil (LPMI-FMUSP).

1.5 - Leishmania (V.) guyanensis

L. guyanensis (MHOM/BR/2001/M19663) from an individual with American tegumentary leishmaniasis in the state of Pará, Brazil. It was classified by isoenzymes in Evandro Chagas Institute, Belém, State of Pará, Brazil. This strain was provided by Luiz Felipe Passero, from *Laboratório de Patologia de Moléstias Infeciosas*, *Faculdade de Medicina da Universidade de São Paulo*, Brazil (LPMI-FMUSP).

2. Cell line

P388D1 is a mouse tumor cell-line, whose cells present macrophage-like characteristics (Koren et al, 1979).

This cell line was cultivated in RPMI (from *Roswell Park Memorial Institute culture medium*) 1640, (Lonza, Belgium) supplemented with 10 % (v/v) of heat-inactivated FBS, 2 mM of L-glutamine (Merck, Germany), 50 $U.mL^{-1}$ (Sigma-Aldrich) and 50 $\mu g.mL^{-1}$ of streptomycin (Sigma-Aldrich). Macrophages were cultivated in suspension at 37ºC in a humidified atmosphere with 5% CO_2.

3. Promastigotes less susceptible to drugs and experimental compounds

3.1 Exposing Leishmania *promastigotes to drug pressure*

To obtain promastigotes more resistant to drugs, two different protocols were used: one protocol were used to generate strains more resistant to Glucantime; the other to originate strains more resistant to miltefosine (MILT), ursolic acid (URS), chalcone-8 (CH8) and quercetine (QC).

Five different species/strains of *Leishmania* were used: *Leishmania infantum* (four passages), *Leishmania amazonensis* HOM (eight passages), *Leishmania amazonensis* PH (eight passages), *Leishmania shawi* (eight passages), *Leishmania guyanensis* (eight passages). A solution of commercial Glucantime® (Merial, France) with 81 $mg.ml^{-1}$ of meglumine antimoniate was used. The concentration of viable P388D1 cells in culture was determined in a Neubauer chamber, after dilution in a solution of Trypan Blue and adjusted to 2×10^6 cells/ml. Cellular suspension (150 µl/well) was added to a 96-well microplate. Glucantime was added to each well in the concentration of 250 $\mu g.ml^{-1}$. The microplate was incubated for 3 h at 37ºC and 5% CO_2. After incubation, the concentration of viable P388D1 cells in each well was determined.

Simultaneously, the concentration of promastigotes in each culture was determined in a Neubauer chamber, after dilution in a solution of RPMI/Glycerol (40%) and adjusted to the triple of P388D1 cells concentration. *Leishmania* suspension (50 µl/well) was added to macrophages. The microplate was incubated for 72 h at 24°C. After this period, the content of each well was collected to a T-Flask and cultivated in Schneider medium with 10% of FBS. When the promastigote concentration in culture reached the initial concentration (determined before the assay), the protocol was repeated with the double of Glucantime concentration. This protocol was used for because the mechanism of action of Glucantime that only works in the intracellular amastigote stage. So, it was used a model of infection with the promastigotes and P388D1. After culture in a T-flask, only the promastigotes that had left the macrophages exposed to Glucantime would grow in a culture medium containing Glucantime.

A different protocol, adapted from (Mateus 2014) was differentiated peomastigotes under miltefosine (MILT), ursolic acid (URS), chalcone-8 (CH8) and quercetine (QC) drug pressure. The following solutions were used: solution of commercial Milteforan® (Virbac, France) with miltefosine (20 $mg.ml^{-1}$); ursolic acid diluted in DMSO (4 $mg.ml^{-1}$), chalcone-8 diluted in DMSO (0.5 $mg.ml^{-1}$) and quercetin diluted in DMSO (9 $mg.ml^{-1}$). The strains used were *L. infantum* (four passages), *L. amazonensis* HOM, *L. amazonensis* PH, *L. shawi* and *L. guyanensis* (twelve passages). The IC_{50} values considered in this work were those referred in Fernandes (2013). High IC_{50} values were excluded and the compounds used for each species/strain are therefore mentioned as: *L. infantum*: miltefosine (INF MILT), ursolic acid (INF URS), chalcone-8 (INF CH8) and quercetine (INF QC); *L. amazonensis* HOM: miltefosine (HOM

MILT) and quercetine (HOM QC); *L. amazonensis* PH: miltefosine (PH MILT), ursolic acid (PH URS), chalcone-8 (PH CH8) and quercetin (PH QC); *L. shawi*: miltefosine (SHAW MILT), ursolic acid (SHAW URS) and chalcone- 8 (SHAW CH8); *L. guyanensis*: miltefosine (GUYA MILT) and ursolic acid (GUYA URS).

For each strain/compound, eight two-fold dilutions were made in a 96-well plate. Replicated three times. In parallel negative controls were made in independent wells without compound addition.

The concentration of each *Leishmania* culture was determined through direct counting in a Neubauer chamber, after dilution in a solution of RPMI/Glycerol (40%) and adjusted to 2×10^6 promastigotes/ml. In each well containing the drug dilutions, 100 µl of adjusted *Leishmania* culture were added. Then, the plates were sealed and incubated for 96 h at 24 °C.

After the period of incubation, the plates were examined by optical microscopy and each well was considered positive or negative according to the presence of live promastigotes or not. The highest dilution in which was possible to find live promastigotes was collected and centrifuged at 130 ×g for 10 min to remove the dead parasites in the medium. The supernatant obtained was centrifuged three times at 1800 ×g for 10 min in sterile saline solution and suspended in complete SCHN medium. The culture was maintained until promastigote concentration reached 2×10^6 promastigotes/ml, where the experiment was repeated. The assay was executed until the drug concentration of the highest dilution in which are present live promastigotes was four times the highest dilution in the first assay (the dilution at the end in which it is possible to find live parasites would be lower than the dilution at the beginning, which means that a higher concentration of compound is needed to kill the same concentration of parasite).

3.2 - Exposing promastigotes to the drug pressure of miltefosine (MILT), ursolic acid (URS), chalcone-8 (CH8) and quercetine (QC) in T-flask culture

The same compounds and the same *Leishmania* strains that are above referred were used in this assay. The concentration of each culture were determined through direct counting in a Neubauer chamber, after dilution in a solution of RPMI/Glycerol (40%) and adjusted to 2×10^6 promastigotes/ml.

In the first round of the experiment, SCHN medium (3ml) present in all cultures contained twice the IC_{50} value according to the compound/species or strain considered

and the DMSO (v/v) were lower than 1%. After the promastigote concentration reached the initial concentration (2 $\times 10^6$ promastigotes/ml), the cultures were centrifuged at 130 ×g during 10 min to remove the dead parasites. Parasite concentration of each culture were determined through direct counting in a Neubauer chamber after dilution in a solution of RPMI/Glycerol (40%) and adjusted to 2 × 10^6 promastigotes/ml. The compounds were again added to each culture, but this time at a higher concentration than before (double IC_{50} concentration). The protocol was successively repeated, but doubling compound concentration in each round (4 times and 8 times the IC50 value).

After centrifugation at 1800 *xg* during 10 min, the pellet obtained for each culture was suspended in PBS and frozen at -80 ºC for later DNA extraction.

4. IC_{50} of experimental compounds in more resistant promastigotes

The IC_{50} values of ursolic acid, chalcone-8 and quercetine were determined for the more resistant *Leishmania* species, previously under drug pressure. The resazurin method that was used in this experiment was adapted from Vale-Costa et al. (2012) and Fernandes (2013). The concentration of promastigotes in all assays was previously adjusted to 5 × 10^5 promastigotes/ml. The concentrations of ursolic acid used ranged between 100 μg.ml^{-1} and 1.56 μg.ml^{-1} , concentrations of chalcone-8 between 10 μg.ml^{-1} and 0.16 μg.ml^{-1} and concentrations of quercetin between 60 μg.ml^{-1} and 0.94 μg.ml^{-1}. The essays were executed in black 96-well microplates (Thermo Fisher Scientific), designed for fluorescence-based assays. Promastigotes with the dilutions of the compounds were incubated for 24 h and then 1.25 mM resazurin/PBS was added to each well. The intensity of fluorescence was read after 4 h. The IC_{10} and IC_{50} for each species were determined in GraphPad.

5. Determination of the effect of antileishmanial compounds combined with efflux pump inhibitors (EPI)

5.1. Infection of P388D1 macrophages (MØ) with Leishmania promastigotes

In a 24-well microplate, it was added a P388D1 macrophage suspension in RPMI 1640 supplemented with 10 % (v/v) of heat-inactivated FBS to each well. The concentration of the suspension was previously adjusted to 8 × 10^5 MØ/ml. More resistant *Leishmania* promastigotes of each species/strain previously developed were

added to each well in a proportion of 3:1. The process of incubation, executed at 37 ºC in a humid atmosphere with 5% of CO_2 had different times according to the *Leishmania* species considered (Table 1).

Species	Incubation time (h)
L. infantum	5
L. amazonensis	48
L. shawi	18
L. guyanensis	18

Table 1. Incubation times for the different *Leishmania* species

5.2. Treatment of infected macrophages with antileishmanial compounds combined with EPI

The concentration of infected macrophages in the previous stage were determined using the trypan blue exclusion method in a Neubauer chamber and then adjusted to 5×10^4 MØ/ml.

The respective IC_{10} value of the EPI (verapamil, VER, sodium orthovanadate, ORT and Phe-Arg β-naphthylamide, PAβN) and the antileishmanial compound was added to each well. IC_{10} values for antileishmanial compounds were obtained in the previous section. IC_{10} values for EPI were based on results of Fernandes (2013) and can be consulted in Table 2.

EPI	**Species**				
	L. infantum	*L. amazonensis* HOM	*L. amazonensis* PH	*L. shawi*	*L. guyanensis*
VER	1.966	1.966	1.966	1.966	1.966
ORT	2.293	1.338	0.829	4.130	1.750
PAβN	0.553	11.478	25.00	10.223	5-887

Table 2. IC_{10} values of the EPI used in this work. All concentrations are expressed in $\mu g.ml^{-1}$.

After this, the cells were incubated for 72 h at 37 ºC in a humid atmosphere with 5% of CO_2.

For each assay, dilutions of non-treated infected macrophages were used as control. Other groups consisted in macrophages treated with the respective

antileishmanial compound, macrophages treated with the respective EPI and macrophages treated with both antileishmanial compounds and EPI. All samples were analyzed in triplicate in a total of three independent assays.

5.3. Limit dilution assay (LDA) to determine the effect of the treatment in the infected macrophages

After the incubation period, the content of each well was centrifuged at 1800 ×g for 10 min. The pellet was ressuspended in SCHN with 10% (v/v) FBS and the macrophage concentration was determined with optical microscopy and adjusted to 5×10^4 MØ/ml. In a 96-well microplate, 200 µl of each suspension was added to the first well and then 8 serial dilutions of 1:4 were made. After 15 days of incubation, each well were observed for the presence or the absence of promastigotes. A well containing a single promastigote was considered positive and a well without any promastigotes negative. All the highest dilutions were compared to the control group to determine the relative percentage of cells that were effectively treated.

5.4 Statistical analysis

The statistical analysis was performed in GraphPad Prism 6.

The control group was compared with the group of macrophages treated with antileishmanial compounds using a nonparametric Mann-Whitney test. A p-value < 0.05 (95%) was considered indicative of statistically significant difference.

The control group was compared with the group of macrophages treated with EPI and the group treated with EPI and antileishmanial compounds. For this purpose, it was performed a nonparametric Kruskal-Wallis test and a Dunn's multiple comparisons test, with a statistical significance of 95 % ($p < 0.05$). In the following Table 3 it is shown the notation used to represent the p-value in graphs.

p value	Notation	Difference
p value ≤ 0.05	*	significant
p value ≤ 0.01	**	Very significant
p value ≤ 0.001	***	Extremely significant
p value ≤ 0.0001	****	Extremely significant

Table 3. Notation for the p-values obtained.

IV. Results

1. Previous exposition to drugs lead to IC50 increases

The values of IC_{50} and IC_{10} for each species/strain of the less susceptible promastigotes were determined and can be observed in Table 3.

Species/strain	*IC_{50} (µg/mol)*	*IC_{10} (µg/mol)*
INF URS	26.4	3.5
INF CH8	3.4	0.8
INF QC	37.7	17.2
HOM QC	9.2	1.9
PH URS	10.6	2.6
PH CH8	5.4	1.5
PH QC	4.8	1.1
SHAW URS	8.3	1.3
SHAW CH8	3.9	0.8
GUY URS	37.5	11.5

Table 4. IC_{50} and IC_{10} values for the various species/strains that were under drug pressure.

From the information on the table, it is possible to conclude that the strains INF CH8 and GUY URS presented the highest IC_{50} values, respectively 37.7 µg.mol^{-1} and 37.5 µg.mol^{-1}. On the other hand, the strains SHAW CH8 and INF CH8 presented the lowest IC_{50} values, respectively 3.8 µg.mol^{-1} and 3.4 µg.mol^{-1}.

Comparing to the IC_{50} values previously obtained for the wild type strains (Fernandes 2013) with the results obtained in the present study show that all strains that

were under drug pressure exhibite higher IC_{50} values, which is a good indicator of their resistance to the tested compounds.

2. Effect of the treatment with antileishmanial compounds combined with EPI in infected macrophages

2.1 – Treatments with URS combined with VER, ORT or PAβN reduce the relative infection rate of INF URS

The group treated with URS and the groups treated with different EPI (VER, ORT, PAβN) did not show any significant differences in the relative infection rate when compared to the control group (Fig.10A, B, C and D). The group simultaneously treated with URS and VER presented a significant reduction (13.3%) in the relative infection rate when compared with control ($p = 0.0005$, Fig. 10B). Both groups treated with URS plus ORT and URS plus PAβN presented significant reductions of the relative infection rate ($p < 0.0001$), respectively 15.0 % and 29.0 % (Fig.10C and D).

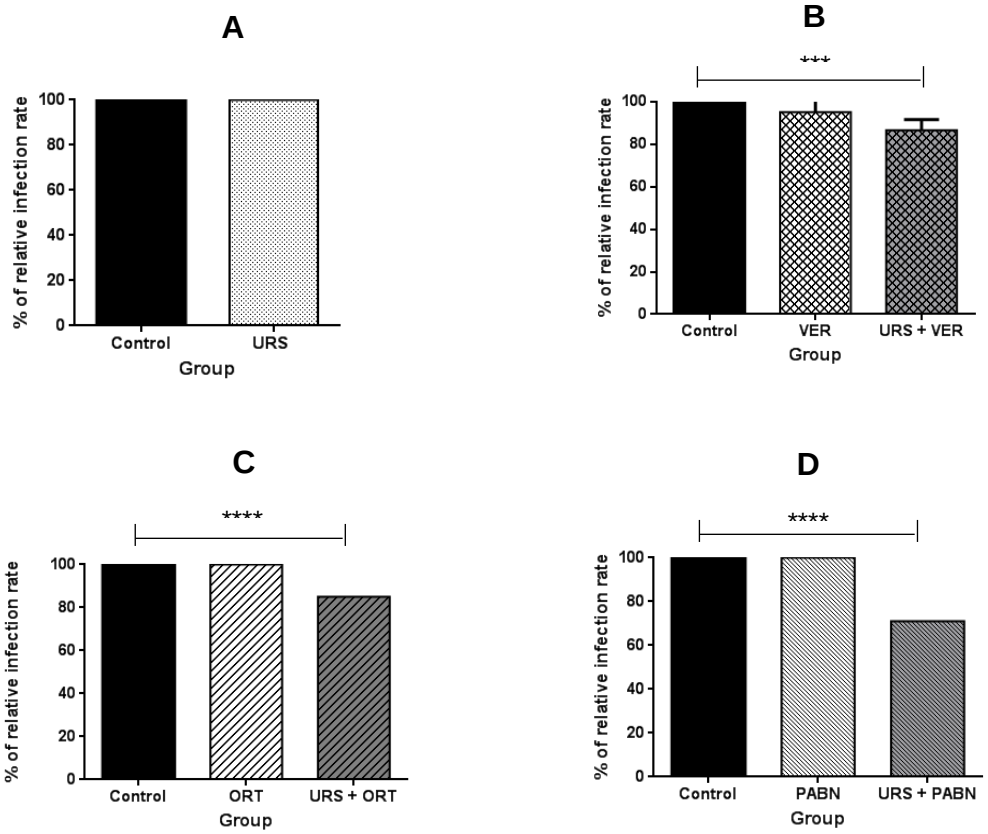

Figure 10. Effect of URS and EPI in macrophages infected with INF URS. The relative infection rate of infected MØ treated with URS (A), VER, URS + VER (B), ORT, URS + ORT (C), PAβN and URS + PAβN (D) was estimated through a dilution limit test. In parallel infected MØ (control) were also evaluated. The results are expressed by the mean and the standard deviation of three independent assays and of three replicates for each condition. *** and **** represent extremely significant differences.

The results suggest that treatments with URS combined with the EPI (VER, ORT or PAβN) reduces the relative infection rate of INF URS, probably by reducing drug efflux.

2.2 – Treatments with CH8 in combination with VER, ORT or PAβN reduce the relative infection rate of INF CH8

Treatment of MØ with CH8 and with the EPIs (VER, ORT, PAβN) did not reduce the relative infection rate (Fig. 11A). Treatment of INF CH8-infected MØ with CH8 in combination with VER reduced significantly (p = 0.0210) the relative infection rate in 7.5% (Fig.11B). When the groups were treated with CH8 plus ORT (Fig. 11C) and CH8 plus PAβN (Fig. 11D) the reduction of parasitized cells was extremely significant, respectively 13.1% (p = 0.003) and 16.5% (p < 0.0001).

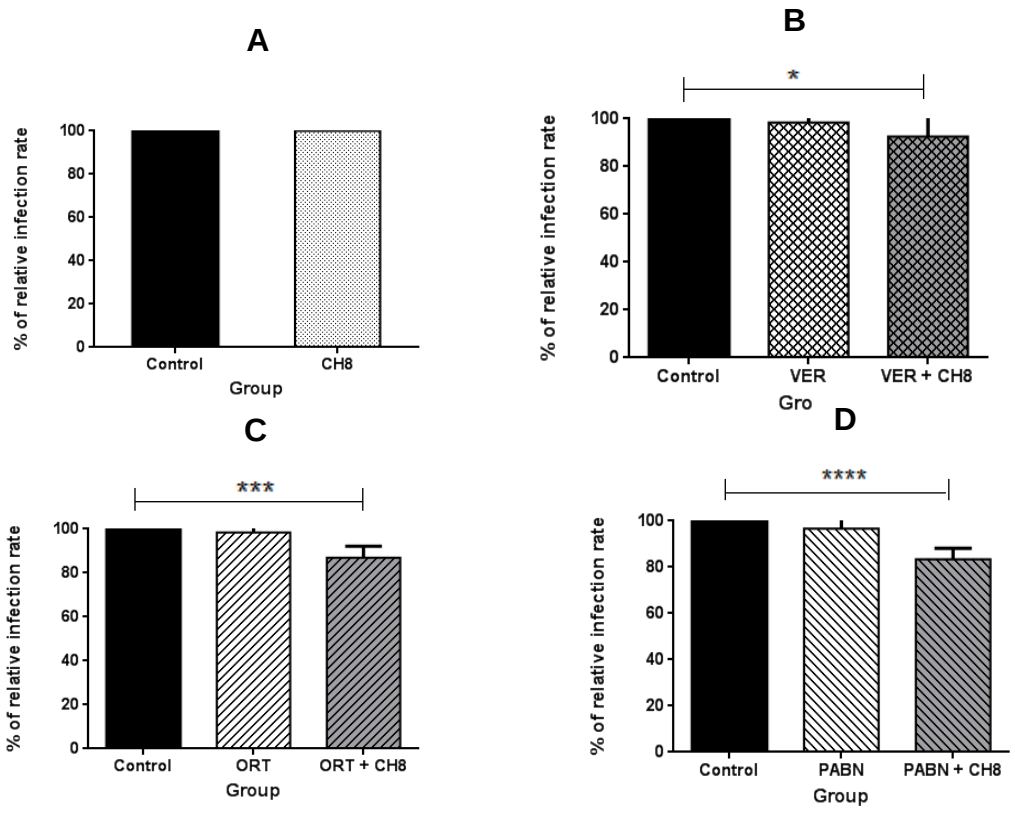

Figure 11. Effect of CH8 and EPI in macrophages infected with INF CH8. The relative infection rate of macrophages treated with CH8 (A), VER, CH8 + VER (B), ORT, CH8 + ORT (C), PAβN and CH8 + PAβN (D) was estimated through a dilution limit test. In parallel infected MØ (control) were also evaluated. The results are expressed by the mean and the standard deviation of three independent assays and of three replicates for each condition. * represents a significant difference, *** and **** represent extremely significant differences.

The results suggest that the treatments with CH8 in combination with VER, ORT or PAβN are able to reduce significantly the relative infection rate of INF CH8, possibly by interfering with the activity of cell transporters.

2.3 - *Treatments with QC in combination with VER, ORT or PAβN significantly reduce the relative infection rate of INF QC*

Treatments of INF QC infect macrophages with QC in combination with EPIs (VER, ORT, PAβN) presented an extremely significant reduction in the relative infection rate of infected macrophages of 16.6% ($p < 0.0001$, Fig. 12B), 13.8% ($p < 0.0001$, Fig. 12C) and 9.7% ($p = 0.0004$, Fig. 12D). Whereas the monotherapy with QC (Fig. 12A) or EPI did not have a significant effect on the relative infection rate.

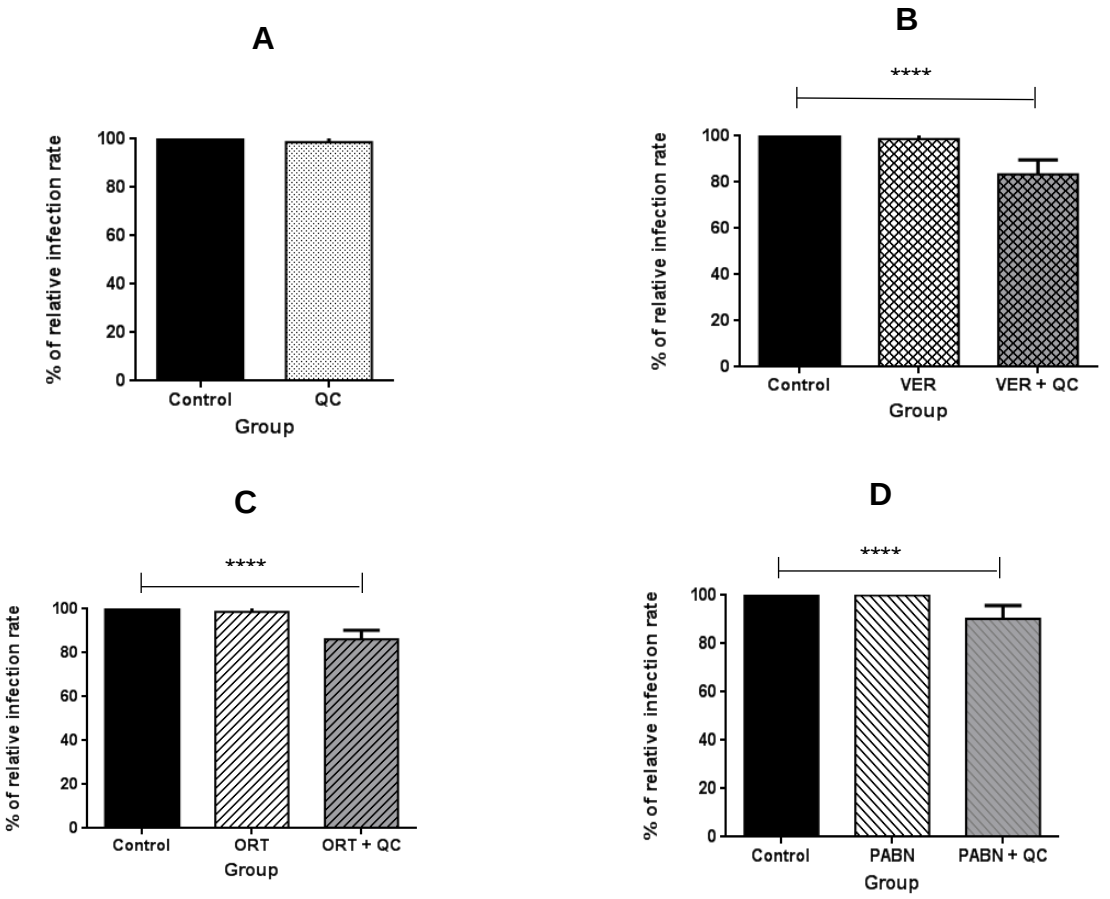

Figure 12. Effect of QC and EPI in macrophages infected with INF QC. The relative infection rate of macrophages treated with QC (A), VER, QC + VER (B), ORT, QC + ORT (C), PAβN and QC + PAβN (D) was estimated through a dilution limit test. In parallel infected MØ (control) were also evaluated. The results are expressed by the mean and the standard deviation of three independent assays and of three replicates for each condition. **** represents extremely significant differences

The results suggest that the treatments with QC and VER, ORT or PAβN are able to reduce significantly the relative infection rate of macrophages infected with INF QC possibly by affecting cell pumps.

2.4 – *Treatments with QC combined with VER or PAβN reduce the relative infection rate of HOM QC*

Treatments of infected macrophages with QC (Fig.13A) or VER (Fig. 13B), ORT (Fig. 13C) and PAβN (Fig. 13D) did not reduce the relative infection rate. Surprisingly, the treatment with QC combined with ORT also did not significantly reduce the relative infection rate. On the other hand, treatment with QC plus VER lead to a high significant reduction (p = 0.0017) in the relative infection rate (10.0%) whereas treatment with QC combined with PAβN lead to an extremely significant reduction of the relative infection rate.

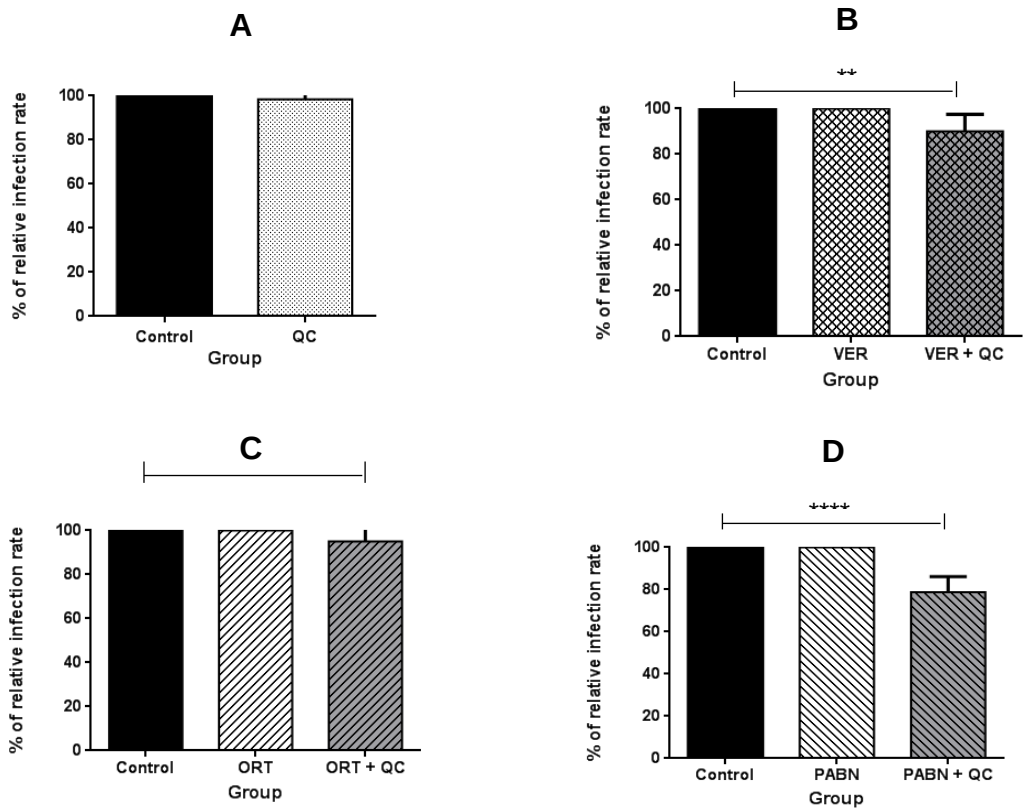

Figure 13. Effect of QC and EPI in macrophages infected with HOM QC. The relative infection rate of macrophages treated with QC (A), VER, QC + VER (B), ORT, QC + ORT (C), PAβN and QC + PAβN (D) was estimated through a dilution limit test. In parallel infected MØ (control) were also evaluated. The results are expressed by the mean and the standard deviation of three independent assays and of three replicates for each condition. ** represents a very significant difference and **** represents an extremely significant difference.

The results suggest that the treatments with QC combined with VER or PAβN are able to reduce significantly the relative infection rate of macrophages infected with HOM QC, suggesting an important role of VER and PAβN in avoiding drug efflux.

2.5 – Treatments with URS combined with VER or PAβN reduce the relative infection rate of PH URS

PH URS infected MØ treated with URS associated with VER (Fig. 14B) and URS combined with PAβN (Fig. 14D) showed very significant reductions in the relative infection rate of 6.9% ($p = 0.0058$) and 9.3% ($p = 0.0012$), respectively. Treatment with URS combined with ORT (Fig. 14C), with EPI (VER, ORT, PAβN) or with URS (Fig. 14A) did not have a significant effect in infected macrophages.

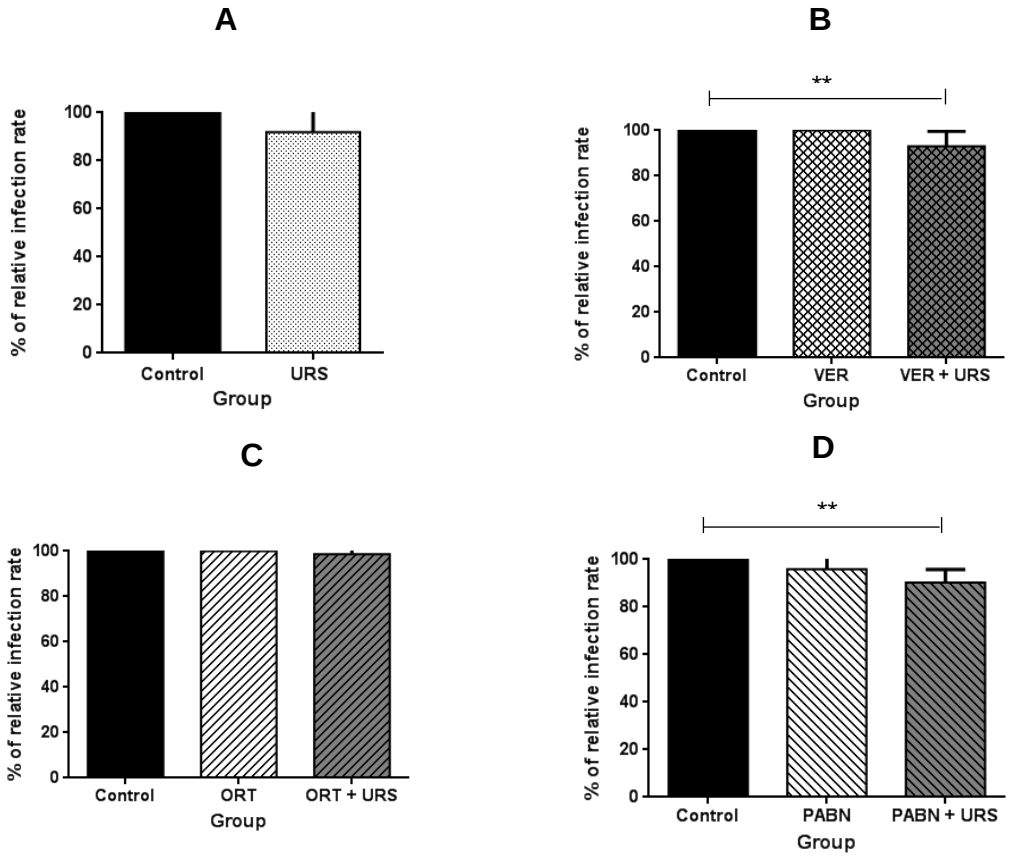

Figure 14. Effect of URS and EPI in macrophages infected with PH URS. The relative infection rate of macrophages treated with URS (A), VER, URS + VER (B), ORT, URS + ORT (C), PAβN and URS + PAβN (D) was estimated through a dilution limit test. In parallel infected MØ (control) were also evaluated. The results are expressed by the mean and the standard deviation of three independent assays and of three replicates for each condition. ** represents very significant differences.

The results show that treatments with URS and VER or PAβN significantly reduce the relative infection rate of macrophages infected with PH URS. Also in this case VER or PAβN seem to have a decisive role in controlling drug efflux.

2.6 –*CH8 reduces the relative infection rate of PH CH8*

PHCH8 infected MØ treated with CH8 in monotherapy presented an extremely significant reduction (p = 0.0004) of the relative infection rate of 22.8% (Fig 15A). A similar reduction (22.6%) was also evidenced by infected MØ treated with CH8 in combination with PAβN (p = 0.0001, Fig. 15D). Treatment with CH8 combined with VER led to a very significant reduction of 10.0% (p = 0.0073). On the opposite side, the infected macrophages treated with CH8 and ORT and exclusively with each EPI (VER, ORT, PAβN) did not exhibit significant differences in infection levels.

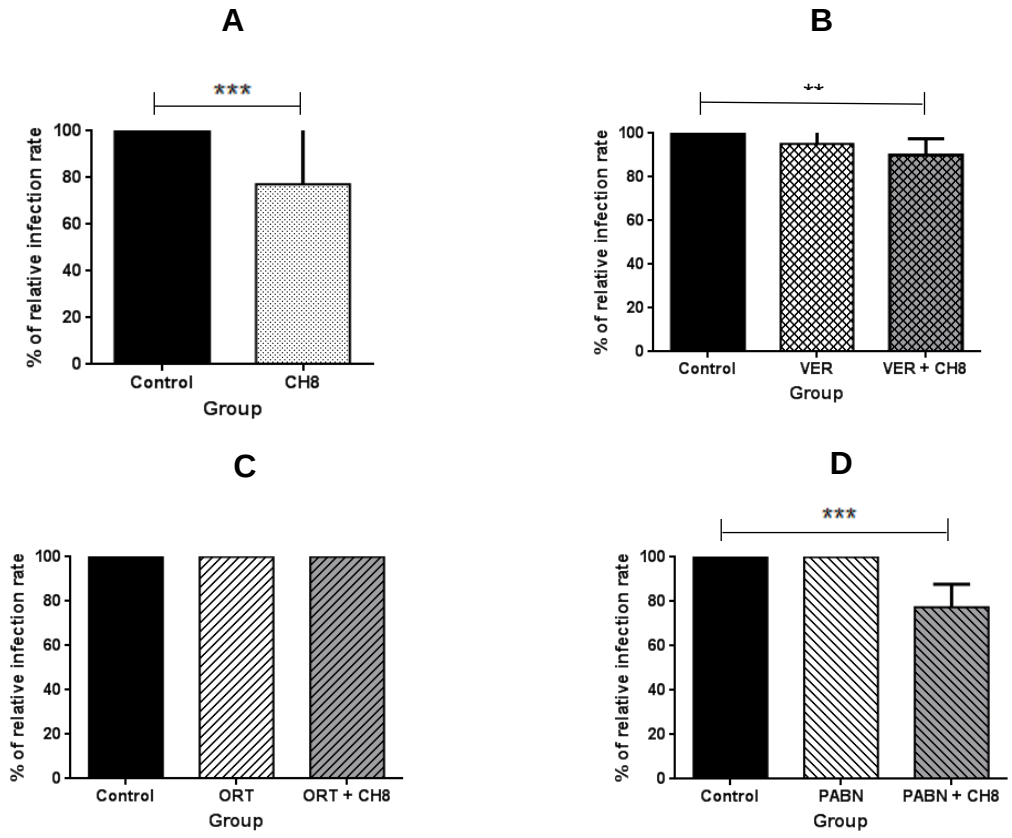

Figure 15. Effect of CH8 and EPI in macrophages infected with PH CH8. The relative infection rate of macrophages treated with CH8 (A), VER, CH8 + VER (B), ORT, CH8 + ORT (C), PAβN, CH8 + PAβN (D) was estimated through a dilution limit test. In parallel infected MØ (control) were also evaluated. The results are expressed by the mean and the standard deviation of three independent assays and of three replicates for each condition. ** represents a very significant difference and *** represents extremely significant differences.

Although CH8 in combination with VER or PAβN significantly reduce the relative infection rate of macrophages infected with PH CH8, the drug alone presents a similar effect. In this case, the effect of EPI seems to be disregarded.

2.7 – *Treatments with QC combined with VER or PAβN reduce the relative infection rate of PH QC*

The treatment of PH QC-infected macrophages with QC combined with VER led to a very significant reduction of 8.3% (p = 0.0058) in the relative infection rate (Fig. 16B) However, the treatment with QC plus PAβN (Fig.16D) caused an extremely significant reduction of 27.4% (p < 0.0001). The exclusive treatment with QC (Fig. 16A), QC in combination with ORT (Fig. 16C) or with EPI (VER, ORT and PAβN) did not affect the relative infection rate.

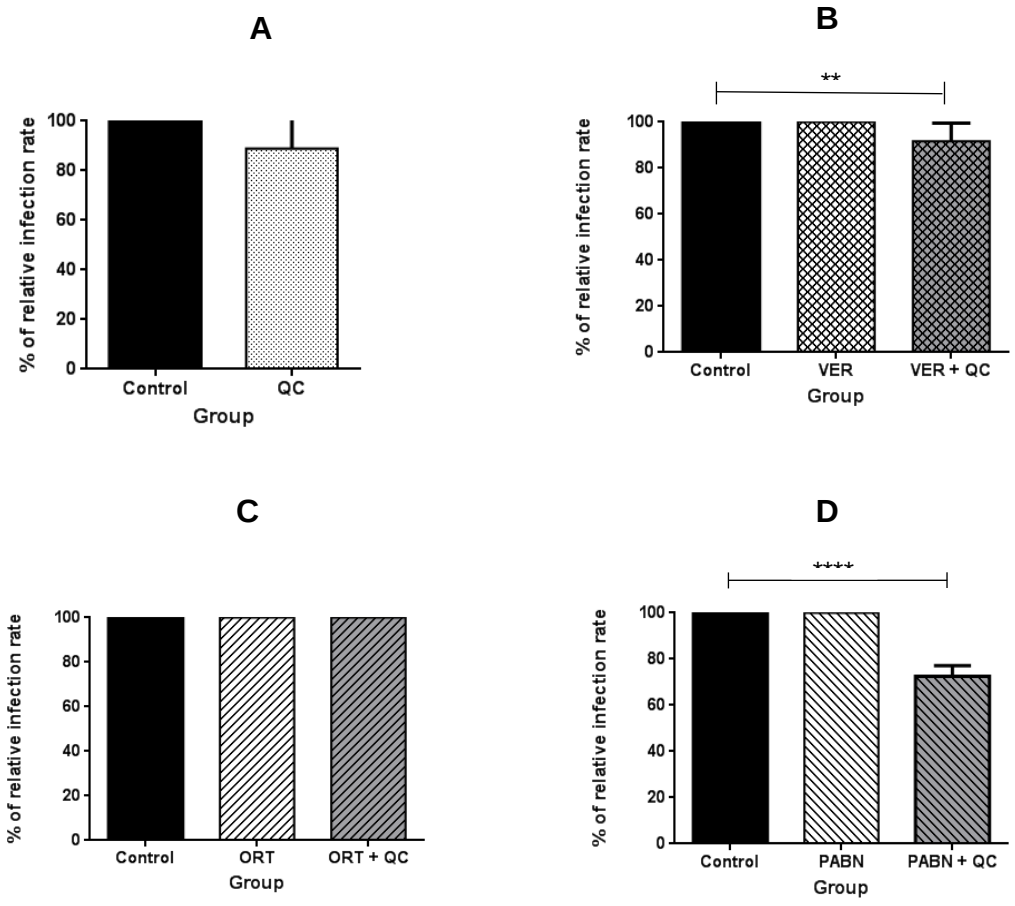

Figure 16. Effect of QC and EPI in macrophages infected with PH QC. The relative infection rate of macrophages treated with QC (A), VER, QC + VER (B), ORT, QC + ORT (C), PAβN and QC + PAβN (D) was estimated through a dilution limit test (LDA). In parallel infected MØ (control) were also evaluated. The results are expressed by the mean and the standard deviation of three independent assays and of three replicates for each condition. ** represents a very significant difference and **** an extremely significant difference.

The results suggest that treatments with QC and VER or PAβN significantly reduce the relative infection rate of macrophages infected with PH QC, pointing towards the activity of these two EPI on pump activity.

2.8 – Treatments with URS in combination with EPI do not reduce the relative infection rate of SHAW URS

The treatment of infected macrophages with URS (Fig. 17A) or with EPI (VER, ORT and PAβN) in monoterapy did not cause a significant reduction of the infection levels. Unlike the expectations, none of the EPI (Fig, 17B, C and D) in combination with URS caused parasite reduction.

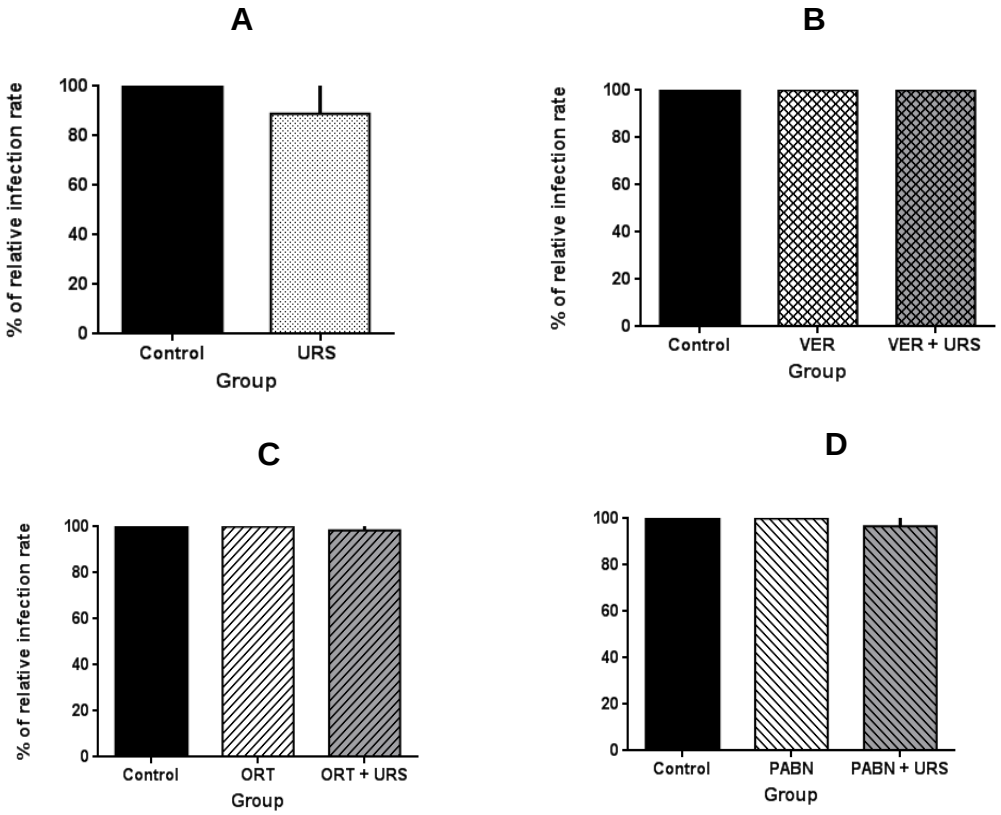

Figure 17. Effect of URS and EPI in macrophages infected with SHAW URS. The relative infection rate macrophages treated with URS (A), VER, URS + VER (B), ORT, URS + ORT (C), PAβN and URS + PAβN (D) was estimated through a dilution limit test (LDA). In parallel infected MØ (control) were also evaluated. The results are the mean and the standard deviation of three independent assays and of three replicate for each condition.

In this case, the use of EPI does not seem to have an effect on SHAW URS parasites, probably due to important changes in parasite cell transporters.

2.9 – *Treatments with CH8 in combination with EPI do not reduce the relative infection rate of SHAW CH8*

SHAW CH8 infected macrophages treated with CH8 in monoterapy or in combination with EPI did not present parasite reduction (Fig.18).

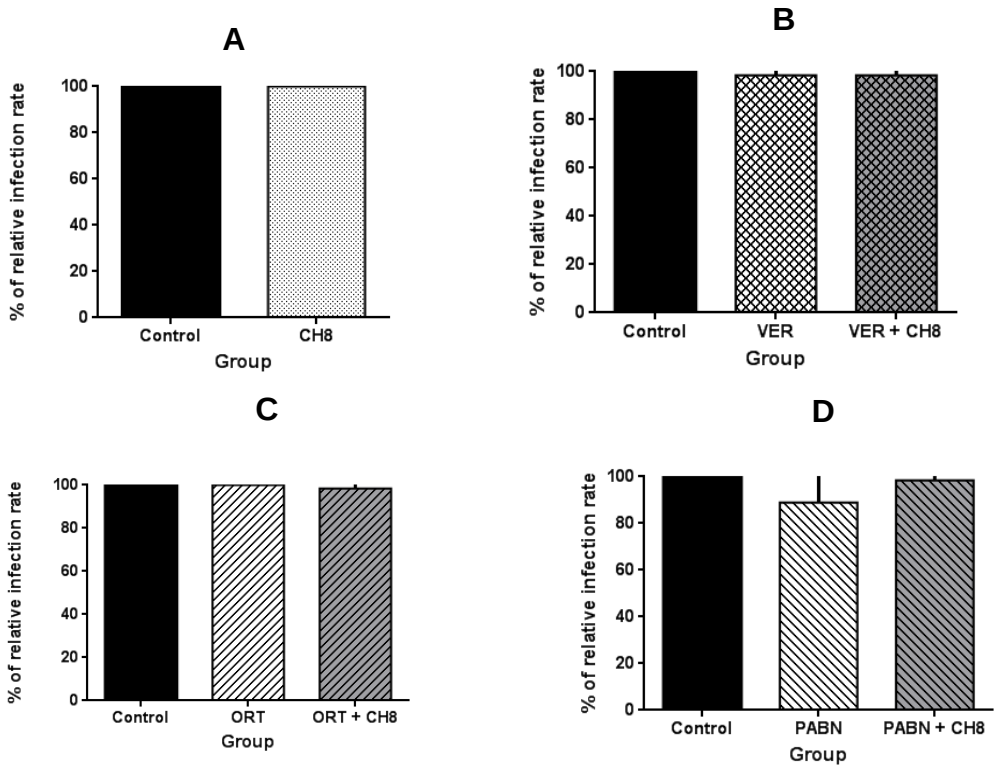

Figure 18. Effect of CH8 and EPI in macrophages infected with SHAW CH8. The relative infection rate of macrophages treated with CH8 (A), VER, CH8 + VER (B), ORT, CH8 + ORT (C), PAβN, CH8 + PAβN (D) was estimated through a dilution limit test (LDA). In parallel infected MØ (control) were also evaluated. The results are expressed by the mean and the standard deviation of three independent assays and of three replicates for each condition.

The results suggest again that the EPI used in the present study do not have a detectable effect on SHAW CH8 parasites, probably by not having effect on drug transport.

2.10 – Treatments with URS in combination with VER, ORT or PAβN do not reduce the relative infection rate of GUYA URS

The relative infection rate of infected macrophages is virtually constant across all groups and the different treatments did not cause an important parasite reduction (Fig.19).

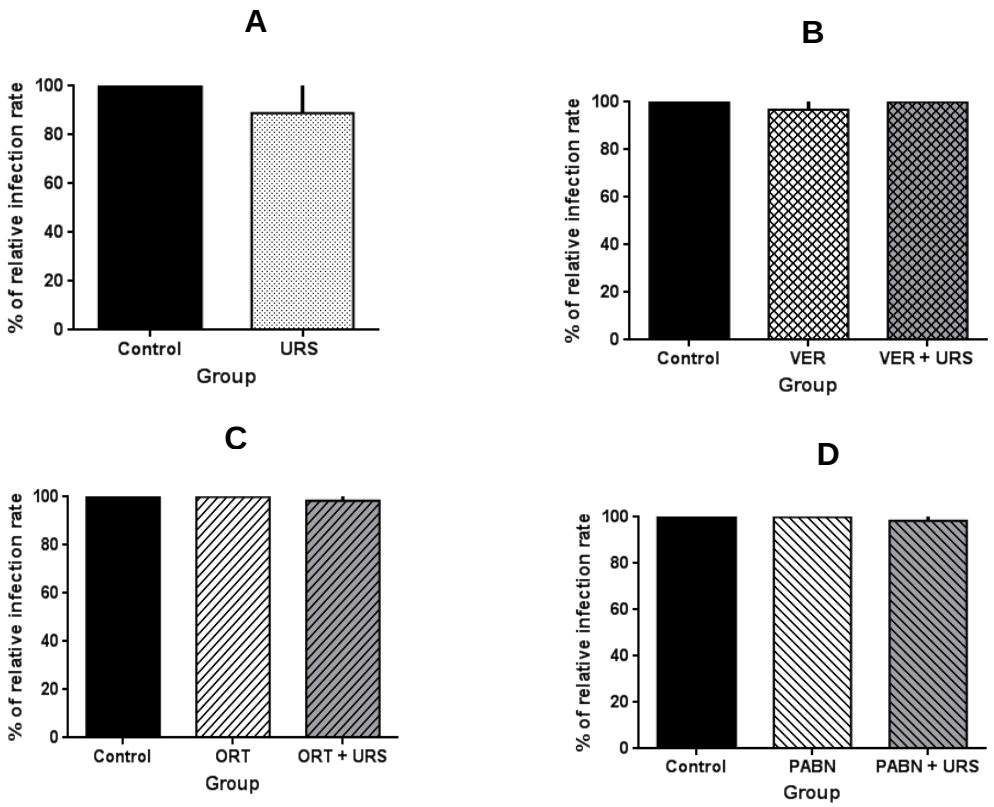

Figure 19. Effect of URS and EPI in macrophages infected with GUYA URS. The relative infection rate of macrophages treated with URS (A), VER, URS + VER (B), ORT, URS + ORT (C), PAβN and URS + PAβN (D) was estimated through a dilution limit test (LDA). In parallel infected MØ (control) were also evaluated. The results are expressed by the mean and the standard deviation of three independent assays with of three replicates for each condition.

The results suggest that treatments with URS in combination with VER, ORT or PAβN do not reduce the relative infection rate of GUYA URS. In this case the effect of EPI in parasite pumps seems to be disregarded.

V. Discussion

Leishmaniasis has a substantial impact all around the world, especially in underdeveloped countries, there are millions of deaths every year but the drugs available to treat the disease are scarce and are continuously losing efficacy. This loss of efficacy occurs mainly because the surge of *Leishmania* strains resistant to the few commercial drugs, so new drugs or new alternatives of treatment are needed to contain the expansion of the disease and treat the existent cases. So, the work aiming the study of drug resistance acquires great significance in the development of new strategies to treat the disease, as Hefnawy and colleagues point out (Hefnawy et al. 2016). Despite this, there is little investment and investigation of alternative treatments for leishmaniasis. The present work tries to contribute to the overcoming of this disappointing scenario.

In terms of drugs, Glucantime is more effective in the amastigote stage of *Leishmania* because it needs the thiol-dependant reductase (TDR1) of the parasite to reduce the Sb(v) to Sb(III), which is the active form of the drug (Denton et al. 2004). Miltefosine acts through the disruption of lipid metabolism (Dorlo et al. 2012b), chalcone acts through the disruption of the parasitic mithocondria (Zhai et al. 1995) and quercetin through the inhibition of parasitic topoisomerase and arginase (Mittra et al. 2000; Da Silva et al. 2012). From this, it's easy to conclude that the antileishmanial compounds need to cross the membrane of the parasite and stay in its interior to execute their function. The reason why the efflux bombs are so important is because they export the compounds to the outside environment, impairing the function of these drugs and so the parasite can thrive even in their presence. The family of ABC transporters are largely responsible for the surge of chemoresistance phenotypes, not only in *Leishmania* parasites (Callahan and Beverley. 1991; Henderson et al. 1992; Katakura et al. 2004; BoseDasgupta et al. 2008; Coelho et al. 2008), but also in other parasitic organisms, as is the case of the helminth *Schistosoma mansoni* (Pinto-Almeida et al. 2015). Besides this, the efflux bombs are associated with antibiotic resistance (Lomovskaya et al. 2001).

According to Ubeda and coworkers, the genetic mechanisms involved in drug resistance in *L. major*, genes that encode for transporter proteins and others, are mainly

gene deletion, the formation of extrachromosomal linear or circular amplicons and aneuploidy, especially with the addition of extrachromosomes (Ubeda et al. 2008).

The strains exposed to drug pressure in this work probably have enhanced efflux pump activity, because the IC_{50} values for ursolic acid, chalcone and quercetine were higher than those observed for the same strains and species, but not resistant (Fernandes. 2013). As the activity of the efflux pumps gets higher, less drugs will remain inside the parasite and less quantity of drug will be available at its site of action. The overall efficacy of the drug diminishes and the survival of the parasites rises. This is an experimental model for what occurs in field resistant-strains, where the activity of efflux pumps impairs the action of the drugs.

One of the mains limitations of the current work lies in the uncertainty about the real resistance to antileishmanial compounds of the promastigotes used in the assays. For a matter of convenience, the term "more resistant" strains is used to define the strains obtained after drug exposure. Because of this, the natural next step of this work would be to look carefully at the expression levels or the mutations of the resistance genes of the *Leishmania* spp promastigotes, such as the following ones: pyridoxal kinase (*PK*) gene, whose mutations are related with appearance of miltefosine resistance in *L. major* promastigotes (Coelho et al. 2012); Ltr ABC1.1 and LtrABC2, whose overexpression are associated with antimoniate resistance (Katakura et al. 2004. Araújo-Santos et al. 2005); PRP1 and MRPA, whose overexpression are presumably responsible for the decrease of influx of antimony in *Leishmania* spp parasites (Ashutosh et al. 2007).

It has been demonstrated that ursolic acid increases nitric oxide production in macrophages (Passero et al. 2011), which leads to the programmed cell death of *Leishmania* parasites (Yamamoto et al. 2014). This compound has shown good antileishmanial activity, for example, in reducing the parasite burden in the spleen and the liver of infected hamsters (Jesus et al. 2017). The association of URS with VER, ORT or PAβN seems to be a good way to make the drug more available inside the parasite, so it can be a promising chemotherapeutic agent in the future.

Transporter proteins that belongs to ABC family are present, not only in the parasitic membrane, but also in the membrane of the macrophage cells. So, the action of the experimental leishmanicidal compounds used in this work can be severely impaired by their acitvity. It's in the overcome of this situation that the action of the EPI can be crucial. VER, for example, it's responsible for P-glycoprotein inhibition in *Leishmania* parasites from several species (Essodaïgui et al. 1999) and for the MDR phenotype reversion in cells (Wu et al. 2014). ORT can revert the MDR phenotype in *Enterecoccus faecalis* (Lee et al. 2003). PAβN it has been used as efflux pump inhibitor in *Escherichia coli* cells (Ospina Barrero et al. 2014).

Previously, the association of VER with Glucantime showed good perspectives in *in vitro* isolates of *L. donovani*, through the reversion of the resistance phenotype (Valiathan et al. 2006) and in *L. tropica* parasites (Shokri et al. 2012). In this work, VER showed that can be administered with antileishmanial compounds, URS, CH8 and QC and can actively increase the efficacy of the drug, reducing the levels of macrophage infection by *L. infantum* and *L. amazonensis*. VER is a calcium channel blocker, so can impair the activity of these kind of efflux pumps of the parasite, and probably of the macrophage, making possible that the drug is not extruded by the parasite or even the macrophage cell. However, it could be the case that, in the infection model that was used, some parasites that appeared in the highest dilution could be outside the macrophages and not be able to enter them, so the combined treatment of the drug with the EPI wouldn't have a direct effect in infection of macrophages. Further works with these compounds need to be done to reassure the efficacy of the treatment.

ORT can significantly reduce infections levels of the macrophages infected with *L. infantum*, but seems to have no effect in other species, like *L. amazonensis*, *L. shawi* and *L. guyanensis*.

PAβN can affect the efflux pump activity and increase the availability of antileishmanial drugs inside the parasite, which corroborates some previous results (Fernandes. 2013). It was the most effective EPI in all species tested.

Since these EPI have little cytotoxicity in macrophage cells and can actively inhibit the activity of the efflux pumps, the use of these compounds simultaneously with

antileishmanial drugs can be an interesting option. The combined treatment with antileishmanial compounds and EPI can effectively reduce the parasitic burden in infected macrophages comparing to monoterapy with antileishmanial compounds. Even so, PAβN does not have a detectable effect on resistant *L. shawi* and *L. guyanensis* parasites, which can be caused by alterations in the expression of the efflux pumps. In these more resistant strains obtained, the amount of ABC transporters present in the parasitic membrane can be higher than the normal in non resistant strains and so the EPI can't affect the activity of all efflux pumps. In the opposite, if these transporters aren't present in the membrane, the EPI will not have any target to act.

Unlike the initial expectations, it was not possible to test the efficacy of the association of the EPI with some conventional leishmanicidal drugs, like Miltefosine and Glucantime. All the essays made in order to obtain susceptible promastigotes to these compounds failed. In the case of Glucantime, the main reason is the excessive drug concentration needed to induce the susceptibility in the promastigotes and the lack of resistance presented after successive rounds of exposure to very high concentrations of this compound. Despite evidence that it is possible to obtain miltefosine-resistant strains of *L. infantum* (Mateus. 2014), it was not possible to obtain the same outcome in the present work, being the reason unknown. Maybe for future works, the protocol can be changed and new ways of inducing resistance in promastigotes can be tested. It would be important to test the association of these commercially available drugs with the EPI, since these two drugs are the ones the public gets access to, whereas the antileishmanial compounds used (URS, CH8 and QC) are only experimental and might never reach the commercialization phase.

Another limitation of the present work it's the fact that all assays were made *in vitro.* It's more difficult to extract conclusions about the real efficacy of this strategy of treatment if the results are not complemented by a set of results obtained from *in vivo* experimental models. As so, one of the future directions this work could take would be to overcome this limitation by executing an array of *in vivo* assays using, for example, hamsters as experimental model. Additionally, other *Leishmania species* than those used in this work can be tested to evaluate the potential of treatment using this new strategy.

VI. Conclusions

The association of VER and PAβN with the experimental antileishmanial compounds URS, CH8 and QC can impair the efflux activity of ABC transporters (both the efflux pumps of the parasite and the macrophage probably), while increasing the availability of the compounds inside the parasites belonging to *L. infantum* and *L. amazonensis* species. ORT is only effective in the treatment of macrophages infected with *L.infantum*. PAβN is the most effective EPI and ORT the less effective.

On the opposite side, the strategy of combining the experimental antileishmanial compounds with EPI seems to be ineffective in macrophages infected with different strains belonging to *L. shawi* and *L. guyanensis* species, which can be explained by alterations in the expression of the efflux pumps in these species. These alterations in the target of the EPI can lead to a loss of efficacy of these compounds.

The positive results open possibilities to execute the strategy of combining antileishmanial compounds with EPI for the treatment of leishmaniasis, but using different antileishmanial compounds, especially conventional drugs, other *Leishmania* species and other resistant strains. The EPI have a significant impact in the decrease of efflux pumps activity and increase of the efficacy of antileishmanial drugs.

VII. References

Afonso MO, Cardoso L, Anastácio S, Janz JG, Semião-Santos S, Sousa S, Rodrigues J, Rodrigues M, Alves-Pires C. 2007. The phlebotomine sandflies of Portugal - IX . Ecology of the leishmaniosis vectors in Alijó Municipality , Alto Douro Region , 2001. Acta Parasitológica Portuguesa, 2007, 14 (1/2): 19-22.

Afonso MO, Campino L, Cortes S, Alves-Pires C. 2005. The phlebotomine sandflies of Portugal. XIII--Occurrence of *Phlebotomus sergenti* Parrot, 1917 in the Arrabida leishmaniasis focus. Parasite, 12(1): 69–72

Afonso MO, Semião-Santos S. 2004. The phlebotomine sand flies of Portugal . XII - The phlebotomine of the Évora leishmaniasis focus, 1999-2000. Acta Parasitológica Portuguesa, 2004, 11 (1-2): 41-45

Alasaad S. 2013. War diseases revealed by the social media: massive leishmaniasis outbreak in the Syrian Spring. Parasites & Vectors, 6: 94

Alawieh A, Musharrafieh U, Jaber A, Berry A, Ghosn N, Bizri AR. 2014. Revisiting leishmaniasis in the time of war: the Syrian conflict and the Lebanese outbreak. International Journal of Infectious Diseases, 29C: 115–119

De Almeida-Amaral EE, Caruso-Neves C , Pires VMP , Meyer-Fernandes JR. 2008. *Leishmania amazonensis*: Characterization of an ouabain-insensitive Na+-ATPase activity. Experimental Parasitology, 118(2):165–171

Alvar J, Vélez ID, Bern C , Herrero M , Desjeux P , Cano J , Jannin J , Boer MD, WHO Leishmaniasis Control Team. 2012. Leishmaniasis worldwide and global estimates of its incidence. PLoS ONE, 7(5): e35671

Alvar J, Aparicio P, Aseffa A, Den Boer M, Cañavate C, Dedet JP, Gradoni L, Ter Horst R, López-Vélez R, Moreno J. 2008. The Relationship between leishmaniasis and AIDS: the Second 10 Years. Clinical Microbiology Reviews, 21(2):.334–359

Alvar J., Croft S., Olliaro P. 2006. Chemotherapy in the treatment and control of leishmaniasis. Advances in Parasitology, 61(05):.223–274.

Alvar J, Yactayo S, Bern, C. 2006. Leishmaniasis and poverty. Trends in Parasitology, 22(12):.552–557.

Amaral L., Viveiros M, Molnar J. 2004. Antimicrobial activity of phenothiazines. *In Vivo*, 18: 725–732.

Araújo-Santos JM, Parodi-Talice A, Castanys S, Gamarro F. 2005. The overexpression of an intracellular ABCA-like transporter alters phospholipid trafficking in *Leishmania*. Biochemical and Biophysical Research Communications, 330(1): 49–55

Ashutosh, Sundar S, Goyal N. 2007. Molecular mechanisms of antimony resistance in *Leishmania*. Journal of Medical Microbiology, 56(PART 2): 143–153.

Bailey M, Lockwood DNJ. 2007. Cutaneous leishmaniasis. Clinics in Dermatology, 25(2),: 203–211

Baiocco P, Colotti G, Franceschini S, Ilari A.. 2009. Molecular basis of antimony treatment in leishmaniasis. Journal of Medicinal Chemistry, 52(8): 2603–2612

Bate P 2007. Transmission of *Leishmania* metacyclic promastigotes by phlebotomine sand flies. International Journal for Parasitology, 37(10): 1097–1106.

Benaim G, Yael GM, Reyes C, Uzcanga G, Figarella K. 2013. Identification of a sphingosine-sensitive Ca^{2+} channel in the plasma membrane of *Leishmania mexicana.* Biochemical and Biophysical Research Communications, 430(3): 1091–6.

Bern C, Maguire JH, Alvar J. 2008. Complexities of assessing the disease burden attributable to leishmaniasis. PLoS Neglected Tropical Diseases, 2(10): e313

Bhardwaj S, Srivastava N, Sudan R, Saha B. 2010. *Leishmania* interferes with host cell signaling to devise a survival strategy. BioMed Research International 2010(2):109189

BoseDasgupta S, Ganguly A, Roy A, Mukherjee T, Majumder HK. 2008. A novel ATP-binding cassette transporter, ABCG6 is involved in chemoresistance of *Leishmania*. Molecular and Biochemical Parasitology, 158(2): 176–188.

Branco S, Alves-Pires, Maia C, Cortes S , Cristovão JMS, Gonçalves L , Campino L, Afonso MO, 2013. Entomological and ecological studies in a new potential zoonotic leishmaniasis focus in Torres Novas municipality, Central Region, Portugal. Acta Tropica, 125(3): 339–348

Callahan HL, Beverley SM. 1991. Heavy metal resistance: a new role for P-glycoproteins in *Leishmania*. The Journal of Biological Chemistry, 266(1990): 18427–18430

Campino L, Maia C, 2010. Epidemiologia das leishmanioses em Portugal. Acta Medica Portuguesa, 23(5): 859–864

Castanys-Muñoz E, Alder-Baerens N, Pomorski T, Gamarro F, Castanys S. 2007. A novel ATP-binding cassette transporter from *Leishmania* is involved in transport of phosphatidylcholine analogues and resistance to alkyl-phospholipids. Mol. Microbiol., 64(5): 1141–1153

Castanys-Muñoz E, Pérez-Victoria JM, Gamarro F, Castanys S. 2008. Characterization of an ABCG-like transporter from the protozoan parasite *Leishmania* with a role in drug resistance and transbilayer lipid movement. Antimicrobial Agents and Chemotherapy, 52(10): 3573–9

Chaara D, Haouas N[2], Dedet JP, Babba H, Pratlong F. 2014. Leishmaniasis in Maghreb: An endemic neglected disease. Acta Tropica, 132(1): 80–93

Chappuis F, Sundar S, Hailu A, Ghalib H, Rijal S, Peeling RW, Alvar J, Boelaert M.. 2007. Visceral leishmaniasis: what are the needs for diagnosis, treatment and control? Nature reviews Microbiology, 5(11): 873–82

Chiquero MJ, Pérez-Victoria JM, O'Valle F, González-Ros JM, Del Moral RG, Ferragut JA, Castanys S, Gamarro F. 1998. Altered drug membrane permeability in a multidrug-resistant *Leishmania tropica* line. Biochemical Pharmacology, 55(2): 131–139

Coelho AC, Gentil LG, Silveira JF, Cotrim PC. 2008. Characterization of *Leishmania (Leishmania) amazonensis* promastigotes resistant to pentamidine. Experimental Parasitology, 120(1): 98–102

Coelho AC, Boisvert S, Mukherjee A, Leprohon P, Corbeil J, Ouellette M. 2012. Multiple mutations in heterogeneous miltefosine-resistant *Leishmania major* population as determined by whole genome sequencing. PLoS Neglected Tropical Diseases, 6(2): e1512

Coelho AC, Messier N, Ouellette M, Cotrim PC. Role of the ABC transporter PRP1 (ABCC7) in pentamidine resistance in *Leishmania* amastigotes. Antimicrobial Agents and Chemotherapy, 51(8): 3030–3032

Coelho AC, Beverley SM, Cotrim PC. 2003. Functional genetic identification of PRP1, an ABC transporter superfamily member conferring pentamidine resistance in *Leishmania major*. Molecular and Biochemical Parasitology, 130(2): 83–90.

Croft SL, Olliaro P. 2011. Leishmaniasis chemotherapy--challenges and opportunities. Clinical Microbiology and Infectious Diseases, 17(10):1478–83.

Croft SL, Sundar S, Fairlamb AH. 2006. Drug resistance in leishmaniasis drugs. Society, 19(1): 111–126.

Cunningham AC. 2002. Parasitic adaptive mechanisms in infection by *Leishmania*. Experimental and Molecular Pathology, 72(2): 132–141

Dantas-Torres F. 2007. The role of dogs as reservoirs of *Leishmania* parasites, with emphasis on *Leishmania (Leishmania) infantum* and *Leishmania (Viannia) braziliensis*. Veterinary Parasitology, 149(3-4): 139–146

Denton H, McGregor JC, Coombs GH. 2004. Reduction of anti-leishmanial pentavalent antimonial drugs by a parasite-specific thiol-dependent reductase, TDR1. The Biochemical Journal, 381(2):405–412

Desjeux P. 2001. The increase in risk factors for leishmaniasis worldwide. Transactions of the Royal Society of Tropical Medicine and Hygiene, 95(3): 239–243

Dodge M, Waller RF, Chow LMC, Zaman MM, Cotton LM, McConville MJ, Wirth DF. 2004. Localization and activity of multidrug resistance protein 1 in the secretory pathway of *Leishmania* parasites. Molecular Microbiology, 51: 1563–1575

Dorlo TP, Balasegaram M, Beijnen JH, de Vries PJ. 2012. Miltefosine: A review of its pharmacology and therapeutic efficacy in the treatment of leishmaniasis. Journal of Antimicrobial Chemotherapy, 67(11): 2576–2597

Dujardin JC, Campino L, Cañavate C, Dedet J, Gradoni L, Soteriadou K, Mazeris A, Ozbel Y, Boelaert M. 2008. Spread of vector-borne diseases and neglect of leishmaniasis, Europe. Emerging Infectious Diseases, 14(7): 1013–1018

Essodaïgui M, Frezard F, Moreira ESA, Garnier-Suillerot A. 1999. Energy-dependent efflux from *Leishmania* promastigotes of substrates of the mammalian multidrug resistance pumps. Molecular and Biochemical Parasitology, 100(1): 73–84

Fernandes MR. 2013. Caracterização da internalização de fármacos antileishmania e novos compostos por macrófagos parasitados por *Leishmania* spp. Repositório Universidade Nova, IHMT: MM - Dissertações de Mestrado. pp152.

Filho AADS, Resende DO, Fukui MJ, Santos FF, Pauletti PM, Cunha WR, Silva ML, Gregório LE, Bastos JK, Nanayakkara NP. et al. 2009. *In vitro* antileishmanial, antiplasmodial and cytotoxic activities of phenolics and triterpenoids from *Baccharis dracunculifolia* D. C. (Asteraceae). Fitoterapia, 80(8): 478–482

Gontijo B, De Carvalho MDLR. 2003. Leishmaniose tegumentar americana. Revista da Sociedade Brasileira de Medicina Tropical, 36(1): 71–80

Gossage SM, Rogers ME, Bates P. 2003. Two separate growth phases during the development of *Leishmania* in sand flies: Implications for understanding the life cycle. International Journal for Parasitology, 33(10): 1027–1034

Grácio MA, Grácio AJS , Viveiros M , Amaral L. 2003. Since phenothiazines alter antibiotic susceptibility of microorganisms by inhibiting efflux pumps, are these agents useful for evaluating similar pumps in phenothiazine-sensitive parasites? International Journal of Antimicrobial Agents, 22(3): 347–351

Guan J, Kyle DE , Gerena L, Zhang Q, Milhous WK , Lin AJ . 2002. Design, synthesis, and evaluation of new chemosensitizers in multi-drug-resistant *Plasmodium falciparum*. Journal of Medicinal Chemistry, 45(13): 2741–2748

Hamill RJ. 2013. Amphotericin B formulations: A comparative review of efficacy and toxicity. Drugs, 73(9): 919–934

Hayani K, Dandashli A, Weisshaar E. 2015. Cutaneous leishmaniasis in Syria: Clinical features, current status and the effects of war. Acta Dermato Venereologica, 95(1): 62–66

Hefnawy A, Berg M, Dujardin J , Muylder GD . 2016. Exploiting knowledge on *Leishmania* drug resistance to support the quest for new drugs. Trends in Parasitology, xx(i):1–13

Henderson DM, Sifri CD, Rodgers M, Wirth DF, Hendrickson N, ULLMAN B. 1992. Multidrug resistance in *Leishmania donovani* is conferred by amplification of a gene

homologous to the mammalian *Mdr1* gene. Molecular and Cellular Biology, 12(6): 2855–2865

Herwaldt B. 1999. Leishmaniasis. Lancet, 354(9185): 1191–1199

Inci R. et al. 2015. Effect of the Syrian civil war on prevalence of cutaneous leishmaniasis in Southeastern Anatolia, Turkey. Medical Science Monitor, 21: 2100–2104

Jacobson RL. 2011. Leishmaniasis in an era of conflict in the Middle East. Vector borne and zoonotic diseases (Larchmont, N.Y.*)*, 11(3): 247–258

Jesus JA, Fragoso TN, Yamamoto ES, Laurenti MD, Silva MS, Ferreira AF, Lago JHG, Santos-Gomes G, Passero LF, 2017. Therapeutic effect of ursolic acid in experimental visceral leishmaniasis. International Journal for Parasitology: Drugs and Drug Resistance, 7(1): 1–11

Katakura K, Fujiseb H, Takedab K, Kanekoc O , Toriic M , Suzukia M, Changd KP, Hashiguchi Y. 2004. Overexpression of LaMDR2, a novel multidrug resistance ATP-binding cassette transporter, causes 5-fluorouracil resistance in *Leishmania amazonensis*. FEBS *Letters*, 561(1-3): 207–212

Katakura K, Iwanami M , Ohtomo H , Fujise H , Hashiguchi Y. 1999. Structural and functional analysis of the LaMDR1 multidrug resistance gene in *Leishmania amazonensis.* Biochem Biophysics Research Communications, 255(2): 289–294

Kevric, Cappel MA, Keeling JH. 2015. New World and Old World *Leishmania* Infections. Dermatologic Clinics, 33(3): 579–593

Killick-Kendrick R. 1990. Phlebotomine vectors of the leishmaniasis: a review. Medical and Veterinary Entomology, 4(1):1–24

Laskay T, Van Zandbergen G, Solbach W. 2003. Neutrophil granulocytes - Trojan horses for *Leishmania major* and other intracellular microbes? Trends in Microbiology, 11(5): 210–214

Lee E, Huda MN, Kuroda T, Mizushima T, Tsuchiya T. 2003. EfrAB, an ABC Multidrug Efflux Pump in *Enterococcus faecalis*. Antimicrobial Agents and Chemotherapy, 47(12): 3733–3738

Légaré D, Richard D, Mukhopadhyay R, Stierhof YD, Rosen BP, Haimeur A, Papadopoulou B, Ouellette M. 2001. The *Leishmania* ATP-binding cassette protein PGPA is an intracellular metal-thiol transporter ATPase. Journal of Biological Chemistry, 276(28): 26301–26307

Leprohon P, Légaré D, Girard I, Papadopoulou B, Ouellette M. 2006. Modulation of *Leishmania* ABC protein gene expression through life stages and among drug-resistant parasites. Eukaryotic Cell, 5(10): 1713–1725

Leroux AE, Krauth-Siegel RL. 2015. Thiol redox biology of trypanosomatids and potential targets for chemotherapy. Molecular and Biochemical Parasitology, 206(1-2):67-74

Liévin-Le MV, Loiseau PM. 2016. *Leishmania* hijacking of the macrophage intracellular compartments. FEBS Journal, 283(4): 598–607

Locher KP. 2009. Structure and mechanism of ATP-binding cassette transporters. Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences, 364(1514): 239–45

Lomovskaya O, Warren MS, Lee A, Galazzo J, Fronko R, Lee M, Blais J, Cho D, Chamberland S, Renau T, Leger R, Hecker S, Watkins W, Hoshino K, Ishida H, Lee VJ.. 2001. Identification and characterization of inhibitors of multidrug resistance efflux pumps in *Pseudomonas aeruginosa* : novel agents for combination therapy identification and characterization of inhibitors of multidrug resistance efflux pumps in *Pseudomonas aeroginosa*. Antimicrobial Agents and Chemotherapy, 45(1): 105–116

Lukes J, Mauricio IL, Schönian G, Dujardin J, Soteriadou K, Dedet J, Kuhls K, Tintaya Q, Jirků M, Chocholová E, Haralambous C, Pratlong F, Oborník M, Horák A, Ayala FJ, Miles MA. 2007. Evolutionary and geographical history of the *Leishmania donovani* complex with a revision of current taxonomy. Proceedings of the National Academy of Sciences of the United States of America, 104(22): 9375–9380

Maia C, Afonso MO, Neto L, Dionísio L, Campino L. 2009. Molecular detection of *Leishmania infantum* in naturally infected *Phlebotomus perniciosus* from Algarve Region, Portugal. Journal of Vector Borne Diseases, 46(4): 268–272

Maroli M, Feliciangeli MD, Bichaud L, Charrel RN, Gradoni L. 2013. Phlebotomine sandflies and the spreading of leishmaniases and other diseases of public health concern. Medical and Veterinary Entomology, 27(2): 123–147

Mateus D. 2014. Genotypic analysis of the *Leishmania infantum* resistance to conventional drugs and new chemically synthesized compounds. Repositório da Universidade de Lisboa, FC - Dissertações de Mestrado. pp 58. 51

Mcgwire BS, Satoskar AR. 2014. Leishmaniasis: Clinical syndromes and treatment. Quarterly Journal of Medicine, 107(1): 7–14

De Mello TFP, Bitencourt HR, Pedroso RB, Aristides SM, Lonardoni MV, Silveira TG.. 2014. Leishmanicidal activity of synthetic chalcones in i. *Experimental* Parasitology, 136(1): 27–34

Menezes JPB, Guedes CES, Petersen ALOA, Fraga DBM, and Veras PST. 2015. Advances in development of new treatment for leishmaniasis. BioMed Research International, 201: 815023

Mittra B, Saha A, Chowdhury AR, Pal C, Mandal S, Mukhopadhyay S, Bandyopadhyay S, Majumder HK.2000. Luteolin, an abundant dietary component is a potent anti-leishmanial agent that acts by inducing topoisomerase II-mediated kinetoplast DNA cleavage leading to apoptosis. Molecular Medicine, 6(6): 527–541.

Moradin N, Descoteaux A., 2012. *Leishmania* promastigotes: building a safe niche within macrophages. Frontiers in Cellular and Infection Microbiology, 2: 121

Murray CJL, Vos T, Lozano R, Naghavi M, Flaxman AD, Michaud C, Ezzati M, Shibuya K, Salomon JA, Abdalla S, Aboyans V, Abraham J, Ackerman I, Aggarwal R, Ahn SY, Ali MK, Alvarado M, Anderson HR, Anderson LM, Andrews KG, Atkinson C, Baddour LM, Bahalim AN, Barker-Collo S, Barrero LH, Bartels DH, Basáñez MG, Baxter A, Bell ML, Benjamin EJ, Bennett D, Bernabé E, Bhalla K, Bhandari B, Bikbov B, Bin Abdulhak A, Birbeck G, Black JA, Blencowe H, Blore JD, Blyth F, Bolliger I, Bonaventure A, Boufous S, Bourne R, Boussinesq M, Braithwaite T, Brayne C, Bridgett L, Brooker S, Brooks P, Brugha TS, Bryan-Hancock C, Bucello C, Buchbinder R, Buckle G, Budke CM, Burch M, Burney P, Burstein R, Calabria B, Campbell B, Canter CE, Carabin H, Carapetis J, Carmona L, Cella C, Charlson F, Chen H, Cheng AT, Chou D, Chugh SS, Coffeng LE, Colan SD, Colquhoun S, Colson KE, Condon J, Connor MD, Cooper LT, Corriere M, Cortinovis M, de Vaccaro KC, Couser W, Cowie BC, Criqui MH, Cross M, Dabhadkar KC, Dahiya M, Dahodwala N, Damsere-Derry J, Danaei G, Davis A, De Leo D, Degenhardt L, Dellavalle R, Delossantos A, Denenberg J, Derrett S, Des Jarlais DC, Dharmaratne SD, Dherani M, Diaz-Torne C, Dolk H, Dorsey ER, Driscoll T, Duber H, Ebel B, Edmond K, Elbaz A, Ali SE, Erskine H, Erwin PJ, Espindola P, Ewoigbokhan SE, Farzadfar F, Feigin V, Felson DT, Ferrari A, Ferri CP, Fèvre EM, Finucane MM, Flaxman S, Flood L, Foreman K, Forouzanfar MH, Fowkes FG, Fransen M, Freeman MK, Gabbe BJ, Gabriel SE, Gakidou E, Ganatra HA, Garcia B, Gaspari F, Gillum RF, Gmel G, Gonzalez-Medina D, Gosselin R, Grainger R, Grant B, Groeger J, Guillemin F, Gunnell D, Gupta R, Haagsma J, Hagan H, Halasa YA, Hall W, Haring D, Haro JM, Harrison JE, Havmoeller R, Hay RJ, Higashi H, Hill C, Hoen B, Hoffman H, Hotez PJ, Hoy D, Huang JJ, Ibeanusi SE, Jacobsen KH, James SL, Jarvis D, Jasrasaria R, Jayaraman S, Johns N, Jonas JB, Karthikeyan G, Kassebaum N, Kawakami N, Keren A, Khoo JP, King CH, Knowlton LM, Kobusingye O, Koranteng A, Krishnamurthi R, Laden F, Lalloo R, Laslett LL, Lathlean T, Leasher JL, Lee YY, Leigh J, Levinson D, Lim SS, Limb E, Lin JK, Lipnick M, Lipshultz SE, Liu W, Loane M, Ohno SL, Lyons R, Mabweijano J, MacIntyre MF, Malekzadeh R, Mallinger L, Manivannan S, Marcenes W, March L, Margolis DJ, Marks GB, Marks

R, Matsumori A, Matzopoulos R, Mayosi BM, McAnulty JH, McDermott MM, McGill N, McGrath J, Medina-Mora ME, Meltzer M, Mensah GA, Merriman TR, Meyer AC, Miglioli V, Miller M, Miller TR, Mitchell PB, Mock C, Mocumbi AO, Moffitt TE, Mokdad AA, Monasta L, Montico M, Moradi-Lakeh M, Moran A, Morawska L, Mori R, Murdoch ME, Mwaniki MK, Naidoo K, Nair MN, Naldi L, Narayan KM, Nelson PK, Nelson RG, Nevitt MC, Newton CR, Nolte S, Norman P, Norman R, O'Donnell M, O'Hanlon S, Olives C, Omer SB, Ortblad K, Osborne R, Ozgediz D, Page A, Pahari B, Pandian JD, Rivero AP, Patten SB, Pearce N, Padilla RP, Perez-Ruiz F, Perico N, Pesudovs K, Phillips D, Phillips MR, Pierce K, Pion S, Polanczyk GV, Polinder S, Pope CA 3rd, Popova S, Porrini E, Pourmalek F, Prince M, Pullan RL, Ramaiah KD, Ranganathan D, Razavi H, Regan M, Rehm JT, Rein DB, Remuzzi G, Richardson K, Rivara FP, Roberts T, Robinson C, De Leòn FR, Ronfani L, Room R, Rosenfeld LC, Rushton L, Sacco RL, Saha S, Sampson U, Sanchez-Riera L, Sanman E, Schwebel DC, Scott JG, Segui-Gomez M, Shahraz S, Shepard DS, Shin H, Shivakoti R, Singh D, Singh GM, Singh JA, Singleton J, Sleet DA, Sliwa K, Smith E, Smith JL, Stapelberg NJ, Steer A, Steiner T, Stolk WA, Stovner LJ, Sudfeld C, Syed S, Tamburlini G, Tavakkoli M, Taylor HR, Taylor JA, Taylor WJ, Thomas B, Thomson WM, Thurston GD, Tleyjeh IM, Tonelli M, Towbin JA, Truelsen T, Tsilimbaris MK, Ubeda C, Undurraga EA, van der Werf MJ, van Os J, Vavilala MS, Venketasubramanian N, Wang M, Wang W, Watt K, Weatherall DJ, Weinstock MA, Weintraub R, Weisskopf MG, Weissman MM, White RA, Whiteford H, Wiebe N, Wiersma ST, Wilkinson JD, Williams HC, Williams SR, Witt E, Wolfe F, Woolf AD, Wulf S, Yeh PH, Zaidi AK, Zheng ZJ, Zonies D, Lopez AD, AlMazroa MA, Memish ZA.. 2012. Disability-adjusted life years (DALYs) for 291 diseases and injuries in 21 regions, 1990-2010: A systematic analysis for the Global Burden of Disease Study 2010. The Lancet, 380(9859): 2197–2223

Neal RA, Bueren JV , McCoy NG, Iwobi M. 1989. Reversal of drug resistance in *Trypanosoma cruzi* and *Leishmania donovan*i by verapamil. Transactions of the Royal Society of Tropical Medicine and Hygiene Royal Society of Tropical Medicine and Hygiene, 83(2): 197–198

Negera E, Gadisa E, Yamuah L, Engers H, Hussein J, Kuru T, Hailu A, Gedamu L, Aseffa A.. 2008. Outbreak of cutaneous leishmaniasis in Silti woreda, Ethiopia: risk

factor assessment and causative agent identification. Transactions of the Royal Society of Tropical Medicine and Hygiene, 102(9): 883–890

Neouiminer N. 1996. Leishmaniasis in the Eastern. Mediterranean Region. Regional Office for the Eastern Mediterranean, 2(1): 94–102

Ospina Barrero MA et al. 2014. Effect of the inhibitors phenylalanine arginyl β-naphthylamide (PAβN) and 1-(1-naphthylmethyl)-piperazine (NMP) on expression of genes in multidrug efflux systems of *Escherichia coli* isolates from bovine mastitis. Research in Veterinary Science, 97(2): 176–181

Ouellette M, Fase-Fowler F, Borst P. 1990. The amplified H circle of methotrexate-resistant *Leishmania tarentolae* contains a novel P-glycoprotein gene. The EMBO Journal, 9(4): 1027–1033

Parodi-Talice A, Araújo JM, Torres C, Pérez-Victoria JM, Gamarro F, Castanys S. 2003. The overexpression of a new ABC transporter in *Leishmania* is related to phospholipid trafficking and reduced infectivity. Biochimica et Biophysica Acta - Biomembranes, 1612(2): 195–207

Passalacqua TG, Dutra LA, Almeida L, Velásquez AMA, Torres FAE, Yamasaki PR,Santos MB. 2015. Synthesis and evaluation of novel prenylated chalcone derivatives as anti-leishmanial and anti-trypanosomal compounds. Bioorganic and Medicinal Chemistry Letters, 25(16): 3342–3345.

Passero LF, Bonfim-Melo A, Corbett CE, Laurenti MD, Toyama MH, de Toyama DO, Romoff P, Fávero OA, dos Grecco SS, Zalewsky CA, Lago JH.. 2011. Anti-leishmanial effects of purified compounds from aerial parts of *Baccharis uncinella* (Asteraceae). Parasitology Research, 108(3): 529–536. 52

Pérez-Victoria JM, Parodi-Talice A, Torres C, Gamarro F, Castanys S. 2001. ABC transporters in the protozoan parasite *Leishmania.* International Microbiology, 4(3): 159–166

Pinto-Almeida A, Mendes T, Armada A, Belo S, Carrilho E, Viveiros M, Afonso A et al. 2015. The Role of Efflux Pumps in *Schistosoma mansoni* Praziquantel Resistant Phenotype. Plos One, 10(10): 0140147

Piscopo TV, Azzopardi CM. 2007. Leishmaniasis. Postgraduate Medical Journal, 83(976): 649–657

Porcheddu A, Giacomelli G, De Luca L. 2012. New pentamidine analogues in medicinal chemistry. Current Medicinal Chemistry, 19(34): 5819–36

Pradines B, Pagès J, Barbe J. 2005. Chemosensitizers in drug transport mechanisms involved in protozoan resistance. Current Drug Targets - Infectious Disorders, 5: 411–431.

Pratlong F, Dereure J, Ravel C, Lami P, Balard Y, Serres G, Lanotte G, Rioux JA, Dedet JP. 2009. Geographical distribution and epidemiological features of Old World cutaneous leishmaniasis foci, based on the isoenzyme analysis of 1048 strains. Tropical Medicine and International Health, 14(9): 1071–1085

Rakotomanga M, Blanc S, Gaudin K, Chaminade P, Loiseau PM. 2007. Miltefosine affects lipid metabolism in *Leishmania donovani* promastigotes. Antimicrobial Agents and Chemotherapy, 51(4):1425–1430

Ready PD. 2013. Biology of phlebotomine sand flies as vectors of disease agents. Annual Review of Entomology, 58: 227–250

Ready PD. 2014. Epidemiology of visceral leishmaniasis. Clinical Epidemiology, 6(1): 147–154

Ready PD. 2010. Leishmaniasis emergence in Europe. European Communicable Disease Bulletin, 15(10): 19505

Reithinger R, Dujardin JC, Louzir H, Pirmez C, Alexander B, Brooker S. 2007. Cutaneous leishmaniasis. The Lancet infectious diseases, 7(9): 581–596

Rittig MG, Bogdan C. 2000. *Leishmania*-host-cell interaction: Complexities and alternative views. Parasitology Today, 16(7): 292–297

Rogan AM, Hamilton TC, Young RC, Klecker RW Jr, Ozols RF. 1984. Reversal of adriamycin resistance by verapamil in human ovarian cancer. Science, 224(1981): 994–996

Roussaki M, Hall, Lima SC, Silva ACV, Wilkinson S, Detsi A. 2013. Synthesis and anti-parasitic activity of a novel quinolinone-chalcone series. Bioorganic and Medicinal Chemistry Letters, 23(23): 6436–6441.

Salotra P, Singh R. 2006. Challenges in the diagnosis of post kala-azar dermal leishmaniasis. Indian Journal of Medical Research, 123: 295–310

Sands M, Kron MA, Brown RB. 1985. Pentamidine: A Review. Reviews of Infectious Diseases, 7(5): 625–634. 53

Sauvage V, Aubert D, Escotte-Binet S, Villena I. 2009. The role of ATP-binding cassette (ABC) proteins in protozoan parasites. Molecular and Biochemical Parasitology, 167(2): 81–94.

Savoia D. 2015. Recent updates and perspectives on leishmaniasis. Journal of Infection in Developing Countries, 9(6): 588–596.

Scalbert A, Williamson G. 2000. Chocolate: Modern science investigates an ancient medicine. Journal of Medicinal Food, 3(2):121–125

Schlein Y. 1993. *Leishmania* and sandflies: Interactions in the life cycle and transmission. Parasitology Today, 9(7): 255–258

Sen G, Mukhopadhyay S, Ray M, Biswas T. 2008. Quercetin interferes with iron metabolism in *Leishmania donovani* and targets ribonucleotide reductase to exert leishmanicidal activity. Journal of Antimicrobial Chemotherapy, 61(5): 1066–1075

Shaw JJ 2002. New world leishmaniasis: the ecology of leishmaniasis and the diversity of leishmanial species in Central and South America. World Class Parasites (4): 11-31

Shokri A, Sharifi I, Khamesipour A, Nakhaee N, Fasihi Harandi M, Nosratabadi J, Hakimi Parizi M, Barati M. 2012. The effect of verapamil on *in vitro* susceptibility of promastigote and amastigote stages of *Leishmania tropica* to meglumine antimoniate. Parasitology Research, 110(3): 1113–1117

Da Silva ER, Maquiaveli CDC, Magalhães PP. 2012. The leishmanicidal flavonols quercetin and quercitrin target *Leishmania (Leishmania) amazonensis* arginase. Experimental Parasitology, 130(3):183–188

Singh N, Chatterjee M, Sundar S. 2014. The overexpression of genes of thiol metabolism contribute to drug resistance in clinical isolates of visceral leishmaniasis (kala azar) in India. Parasites & Vectors, 7(1):1–11

Singh VP, Ranjan A, Topno RK, Verma RB, Siddique NA, Ravidas VN, Kumar N, Pandey K, Das P. 2010. Estimation of under-reporting of visceral leishmaniasis cases in Bihar, India. American Journal of Tropical Medicine and Hygiene, 82(1): 9–11

Soto J, Arana BA, Toledo J, Rizzo N, Vega JC, Diaz A, Luz M, Gutierrez P, Arboleda M, Berman JD, Junge K, Engel J, Sindermann H. 2004. Miltefosine for new world cutaneous leishmaniasis. Clinical Infectious Diseases, 38(9): 1266–1272

Sun EW, Shi YF. 2001. Apoptosis: The quiet death silences the immune system. Pharmacology and Therapeutics, 92(2-3):135–145

Sundar S, More DK, Singh MK, Singh VP, Sharma S, Makharia A, Kumar PCk, Murray HW. 2000. Failure of pentavalent antimony in visceral leishmaniasis in India:

report from the center of the Indian epidemic. Clinical Infectious Diseases, 31(4): 1104–1107

Torres-santos EC, Moreira DL, Kaplan MA, Meirelles MN, Rossi-Bergmann B. 1999. Selective effect of 2',6'-dihydroxy-4'-methoxychalcone isolated from *Piper aduncum* on *Leishmania amazonensis*. Antimicrobial Agents and Chemotherapy, 435(5): 1234–1241

Tsuruo T, Iida H, Tsukagoshi S, Sakurai Y. 1981. Overcoming of vincristine resistance in P388 leukemia *in vivo* and *in vitro* through enhanced cytotoxicity of vincristine and vinblastine by verapamil. Cancer Research, 41(5): 967–1972

Ubeda JM, Légaré D, Raymond F, Ouameur AA, Boisvert S, Rigault P, Corbeil J, Tremblay MJ, Olivier M, Papadopoulou B, Ouellette M 2008. Modulation of gene expression in drug resistant *Leishmania* is associated with gene amplification, gene deletion and chromosome aneuploidy. Genome Biology, 9(7): R115

Vale-Costa S, Vale N, Matos J, Tomás A, Moreira R, Gomes P, Gomes MS 2012. Peptidomimetic and organometallic derivatives of primaquine active against *Leishmania infantum*. Antimicrobial Agents and Chemotherapy, 56(11): 5774–5781

Valiathan R, Dubey ML, Mahajan RC, Malla N. 2006. *Leishmania donovani*: effect of verapamil on *in vitro* susceptibility of promastigote and amastigote stages of Indian clinical isolates to sodium stibogluconate. Experimental Parasitology, 114(2): 103–8

Wang X, Zhang F, Yang L, Mei Y, Long H, Zhang X, Zhang J, Qimuge-Suyila, Su X. 2011. Ursolic acid inhibits proliferation and induces apoptosis of cancer cells i*n vitro* and i*n vivo*. Journal of Biomedicine & Biotechnology, 2011: 419343

WHO. 2010. Control of the leishmaniases. World Health Organization Technical Teport Series, (949): 22–26

WHO. 2015. Kala-azar elimination programme. Report of a WHO consultation of partners Geneva, Switzerland. Accesible in http://www.who.int/neglected_diseases/resources/9789241509497/en/

WHO. 2016. Leishmaniasis and HIV coinfection. Leishmaniasis. Acccessible in http://www.who.int/leishmaniasis/burden/hiv_coinfection/burden_hiv_coinfection/en/

WHO. 2013. Sustaining the drive to overcome the global impact of neglected tropical diseases. Second WHO report on neglected tropical diseases, 3.9:.67–71

Witko-Sarsat V, Rieu P, Descamps-Latscha B, Lesavre P, Halbwachs-Mecarelli L. 2000. Neutrophils: molecules, functions and pathophysiological aspects. Laboratory Investigation, 80(5): 617–653

Wu JQ, Shao K, Wang X, Wang R, Cao Y, Yu Y, Lou J, Chen Y, Zhao H, Zhang Q, Weng X, Jiang C, Zhu L. 2014. *In vitro* and *in vivo* evidence for amphotericin B as a P-glycoprotein substrate on the blood-brain barrier. Antimicrobial Agents and Chemotherapy, 58 (8): 4464-4469

Wyllie S, Cunningham ML, Fairlamb AH. 2004. Dual action of antimonial drugs on thiol redox metabolism in the human pathogen *Leishmania donovani.* Journal of Biological Chemistry, 279(38): 39925–39932

Yamamoto ES, Campos BLS, Jesus JA, Laurenti MD, Ribeiro SP, Kallás EG, Fernandes MR, Santos-Gomes G, Silva MS, Sessa DP, Lago JHG, Levy D, Passero LF. 2015. The effect of ursolic acid on *Leishmania (Leishmania) amazonensis* is related to programed cell death and presents therapeutic potential in experimental cutaneous leishmaniasis. PLoS ONE, 10(12): 1–19

Yamamoto ES, Campos BL, Laurenti MD, Lago JH, Grecco Sdos S, Corbett CE, Passero LF. 2014. Treatment with triterpenic fraction purified from *Baccharis uncinella* leaves inhibits *Leishmania (Leishmania) amazonensis* spreading and improves Th1 immune response in infected mice. Parasitology Research, 113(1): 333–339

Van Zandbergen G, Hermann N, Laufs H, Solbach W, Laskay T. 2002. *Leishmania* promastigotes release a granulocyte chemotactic factor and induce interleukin-8 release but inhibit gamma interferon-inducible protein 10 production by neutrophil granulocytes. Infection and Immunity, 70(8): 4177–4184

Zhai L, Blom J, Chen M, Christensen SB, Kharazmi A. 1995. The antileishmanial agent licochalcone A interferes with the function of parasite mitochondria. Antimicrobial Agents and Chemotherapy, 39(12): 2742–2748